2011

Genetic Manipulation of the Relapsing Fever Spirochete Borrelia hermsii Permits Novel Investigation into the Role of Factor H Binding in Borrelial Virulence

Lindy Fine
Virginia Commonwealth University

Genetic Manipulation of the Relapsing Fever Spirochete *Borrelia hermsii* Permits Novel Investigation into the Role of Factor H Binding in Borrelial Virulence

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by
Lindy M. Fine
Bachelor of Arts, St. Mary's College of Maryland, 2002

Director: Richard T. Marconi, Ph.D.
Professor, Department of Microbiology and Immunology

Virginia Commonwealth University
Richmond, Virginia
June, 2011

Acknowledgement

I would like to dedicate this work to my family:
Jack, my husband and partner in all things;
my loving and supportive parents, Maureen and Gary;
and my best four-legged friends, Girl and Eve, who always remind me
what's most important.

Table of Contents

List of Tables

Page

List of Figures

Page

List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ALBI	affinity ligand binding immunoblot
B. afzelii	*Borrelia afzelii*
B. andersonii	*Borrelia andersonii*
B. burgdorferi	*Borrelia burgdorferi*
B. crocidurae	*Borrelia crocidurae*
B. duttonii	*Borrelia duttonii*
B. garinii	*Borrelia garinii*
B. hermsii	*Borrelia hermsii*
B. hispanica	*Borrelia hispanica*
B. parkeri	*Borrelia parkeri*
B. persica	*Borrelia persica*
B. recurrentis	*Borrelia recurrentis*
B. turicatae	*Borrelia turicatae*
BSA	bovine serum albumin
BSK	Barbour-Stoenner-Kelly
bp	base pair
CHEF	contour-clamped homogeneous electric field
cp	circular plasmid
DF	darkfield microscopy
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FH	factor H
FhbA	factor H-binding protein A
FHBP	factor H-binding protein
FHL-1	factor H-like protein 1
FI	factor I
FlaB	flagellin B
FM	fluorescence microscopy
g	gravity
GFP	green fluorescent protein
GG	genomic group
HRP	horseradish peroxidase
IFA	immunofluorescence assay
IgG	immunoglobulin G

IgM	immunoglobulin M
IL	interleukin
IPTG	isopropylthio-β-D-galactoside
J-HR	Jarisch-Herxheimer reaction
kan	kanamycin
kb	kilobase
kDa	kilodalton
LBRF	louse-borne relapsing fever
LD	Lyme disease
lp	linear plasmid
M	molar
mg	milligram
ml	milliliter
mM	millimolar
ng	nanograms
O. sonrai	*Ornithodoros sonrai*
O. turicata	*Ornithodoros turicata*
O. moubata	*Ornithodoros moubata*
O. hermsi	*Ornithodoros hermsi*
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
PMSF	phenylmethanesulfonylfluoride
PVDF	polyvinylidene diflouride
RF	relapsing fever
SCR	short consensus repeat
SDS	sodium dodecyl sulfate
TBRF	tick-borne relapsing fever
V	volts
Vlp	variable large protein
Vsp	variable small protein
Vtp	variable tick protein
UV	ultraviolet
µg	microgram
µl	microliter
µM	micromolar
°C	degrees Celsius

Abstract

GENETIC MANIPULATION OF THE RELAPSING FEVER SPIROCHETE *BORRELIA HERMSII* PERMITS NOVEL INVESTIGATION INTO THE ROLE OF FACTOR H BINDING IN BORRELIAL VIRULENCE

By Lindy M. Fine, B.A.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Director: Richard T. Marconi, Ph.D.
Professor, Department of Microbiology and Immunology

Borrelia hermsii, an etiologic agent of tick-borne relapsing fever, binds negative complement regulator factor H (FH) via its FhbA protein. Direct demonstration of the role of FhbA in the disease process has been hindered by the lack of genetic manipulation systems for the relapsing fever *Borrelia*. Here, we demonstrate successful generation of a *B. hermsii* strain YOR *fhbA* deletion mutant (*Bh* YOR∆*fhbA*) that constitutively produces green fluorescent protein (GFP). Genetic manipulation did not affect growth rate or plasmid composition. *Bh* YOR∆*fhbA* lost factor H-binding and C3b-inactivation capabilities, but retained resistance to killing in human serum and infectivity in mice. Stable production of GFP was demonstrated *in vitro* and *in vivo*. Collectively, these results suggest that *B. hermsii* employs an unidentified mechanism of complement evasion that is FH-independent and sufficient for persistence within the host. Additionally, this study represents a significant methodological advancement in the

molecular characterization of relapsing fever spirochetes.

Introduction

Borrelia and human health

Pathogenic *Borrelia* species include the causative agents of Lyme disease and relapsing fever (14, 157), earning the genus an important place in the history of public health. Lyme disease (LD) is a significant infectious disease of modern times, and has undergone marked range expansion since it was first recognized in the United States several decades ago (71, 158). Currently, Lyme disease, caused by *B. burgdorferi* in North America and *B. burgdorferi*, *B. garinii*, and *B. afzelii* in Europe (7, 97), is the most common tick-borne disease in the Northern Hemisphere (32, 156, 174).

Relapsing fever (RF) is an ancient disease that was documented during the time of Hippocrates. However, it was not until 1873 that a spirochetal etiologic agent of the louse-borne form of relapsing fever was identified. In the centuries surrounding this discovery, epidemics of louse-borne relapsing fever (LBRF) devastated populations worldwide (24). Most widespread outbreaks of the 20th century were associated with wartime (the World Wars in particular) and ensuing conditions of poverty and poor sanitation. In the first half of the century, there were millions of cases of relapsing fever in Africa and the Balkans, and the disease caused more than 5 million deaths in Russia alone (24, 42, 126). Today, epidemic LBRF remains largely confined to northeastern Africa. Ethiopia is a focal point of infection and reports approximately 10,000 cases per year (24).

Not attributed to the spirochete until 1904 (133), tick-borne relapsing fever (TBRF) has had a more understated, but equally persistent, role in human history as an endemic disease regionally affecting most of the world aside from Australia, New Zealand, and Oceania (55). Today, TBRF remains a significant human health concern. Its impact is most dramatically felt in parts of Africa (Tanzania, Ethiopia, Senegal, Mauritania, and Mali) (41, 45, 82) where it is the most common cause of fever, surpassing even malaria, in certain regions (168). In the heavily impacted Tanzanian village of Mvumi, TBRF sickens nearly 40% of children less than one year of age and is the seventh most frequent cause of childhood mortality (164). Complicating the present situation, TBRF is not a reportable disease in many African countries; thus, the current epidemiology of this disease is poorly understood. A 2004 study underscored this point by revealing that 8.8% of "fever" patients in Togo in fact suffered from TBRF, a disease unknown in that country previously (116). TBRF is also endemic to regions of North America, Mexico, Central and South America, the Mediterranean, and Central Asia (48). In North America, TBRF occurs sporadically west of the Mississippi River in the United States, as well as in southern British Columbia, Canada (22, 47, 49, 62, 144, 148, 155, 167).

Etiology and transmission of relapsing fever

Relapsing fever is caused by at least 15 different *Borrelia* species (127, 130) that are transmitted to humans by arthropod vectors. LBRF is distinguished from other forms of the disease by several unique etiological features. It is caused exclusively by the spirochete *B. recurrentis*, and is transmitted by the human body louse, *Pediculus humanus humanus* (126). Infection with LBRF does not occur through bites of feeding arthropods, but rather through autoinoculation of abraded skin with infected louse hemolymph and possibly feces (75). Spread

of this disease is highly dependent upon human-louse association for several reasons. First, LBRF is not a zoonosis; rather, humans act as the sole mammalian host (14). Secondly, because transovarial transmission of spirochetes does not occur in lice, the naive louse may become infected only through immediate interaction with a spirochetemic human (55).

In contrast, TBRF is caused by a number of different Borrelial species, including the Old World TBRF spirochetes *B. duttonii, B. crocidurae,* and *B. persica*, and the North American spirochetes *B. hermsii* and *B. turicatae* (54, 67). All are transmitted to humans through bites of infected argasid *Ornithodoros* ticks. Because TBRF spirochetes are present in tick salivary glands, direct transmission to the host occurs even during the alacritous (20 min-1 hr) bloodmeals typical of soft-bodied ticks (143). The bacterium-vector relationship for TBRF spirochetes is thought to be highly specific—generally each *Borrelia* species associates with a single vector, and vice versa (e.g., *B. crocidurae* is transmitted only by *O. sonrai, B. turicatae* only by *O. turicata,* etc.) (169). Many TBRF vectors are long-lived and capable of transmitting infection transovarially, making them efficient Borrelial reservoirs and lessening the ecological reliance of many TBRF spirochetes on vertebrate hosts (55).

TBRF is generally an enzootic disease of which humans are accidental hosts. The exception to this is *B. duttonii*, transmitted by *O. moubata*, for which no natural hosts other than humans have been identified (55). Other animals important in the TBRF enzootic cycle include small mammals (particularly rodents), and possibly reptiles, birds, and bats (52, 58, 135, 145, 147, 162). Cases of relapsing fever in domestic dogs and horses have also been documented (129, 146, 171).

In North America, TBRF is most often caused by *B. hermsii*. The tick vector, *O. hermsi*, is primarily encountered in the coniferous, high-elevation forests of the western United States.

Pine squirrels (*Tamiasciurus* spp.) and chipmunks (*Tamias* spp.) are important natural hosts for *B. hermsii*, and the majority of human cases occur in individuals vacationing in rustic cabins that provide a natural interface with both rodent and tick (25, 46, 148).

Clinical disease

Relapsing fever is characterized by recurring episodes of high fever that coincide with the occurrence of large numbers of spirochetes in the blood (54). Manifestations of TBRF and LBRF in humans are very similar, although the latter tends to cause more severe disease. After an incubation period of 2-18 days, illness begins abruptly with nondescript, flu-like symptoms such as fever, headache, myalgia, and arthralgia. Fever (often $\geq$ 104°F) may be accompanied by vomiting, dizziness, or delirium. Tachycardia, hepatomegaly (with or without jaundice), and splenomegaly are commonly observed (154). LBRF is sometimes associated with hemorrhagic phenomena such as the development of petechiae and/or epistaxis (25-50% of cases). A variety of respiratory, cardiac, neurological, and ocular complications occur less commonly (126). Without treatment, symptoms persist for 2-7 days until the individual generates an antibody response sufficient to cause mass clearance of circulating organisms. This results in liberation of bacterial components in the bloodstream and subsequent host release of pro-inflammatory cytokines, causing a crisis of rigors, hypotension, and diaphoresis. Cardiovascular collapse occasionally results (46), and the majority of deaths occur during this phase of infection. The crisis is followed by temporary symptom resolution and spirochetemic relapses of decreasing severity.

Diagnosis is typically made by microscopic observations of spirochetes in the patient's blood (44). However, this method requires that blood be examined during febrile peaks, and even

then spirochetes will be present in only about 70% of samples (154). A more sensitive method involves recultivation of spirochetes from patients' blood either in liquid culture or through inoculation of susceptible laboratory animals (46, 154). Serological or PCR-based methods are occasionally used in research settings or in isolated clinical cases.

Treatment of relapsing fever consists of antibiotics (doxycycline, erythromycin, or chloramphenical) administered over the course of seven days (44). Antibiotic administration often precipitates a Jarisch-Herxheimer reaction (J-HR), which is akin to the crisis phase of natural infection and similar to the classic endotoxin reaction. *Borrelia* lack LPS (see next section), but actions of the antigenic variable major proteins (Vsp/Vlp) have been implicated in this inflammatory process (149, 170). Within the host, J-HR is associated with increased plasma levels of IL-6, IL-8, and TNFα (114), and pre-treating patients with anti-TNFα antibodies has been shown to attenuate the reaction's severity (53).

Without treatment, case mortality rates range from 4-10% for TBRF and 10-40% for LBRF; antibiotic therapy decreases these numbers to <5% (126). Much higher rates of death (up to 70% (24)) were documented during past epidemics, likely a result of malnourishment and generalized poor health among the affected populations.

Cell structure and genetics

The *Borrelia* are variably sized spirochetes, ranging from 8-30 μm in length and from 0.2-0.5 μm in width (14). They have a diderm membrane ultrastructure (85) but, unlike traditional Gram-negative organisms, lack lipopolysaccharide (LPS) (163). Between the outer membrane sheath and the protoplasmic cylinder, *Borrelia* have a variable number (7-30) of periplasmic flagella organized in flat, ribbon-like formations (14, 34). Interactions between the

flagella and the cell cylinder confer upon Borrelial spirochetes a distinctive flat-wave morphology and translational motion (66).

The *Borrelia* have an extremely unusual, fragmented genomic structure consisting of a linear chromosome and multiple linear and circular plasmids (10, 14, 16, 56). This unique genomic composition, dominated by extrachromosomal elements and covalently-closed linear DNA molecules (13, 33), appears to be ubiquitous among both Lyme and RF spirochetes (30, 83, 92). The linear chromosome of *B. burgdorferi* type strain B31 has a G+C content of 28.6% and bears most housekeeping genes amidst the compactly arranged coding sequences that collectively occupy 93% of the chromosome (60). Additional sequencing data have suggested that this organization is generally conserved among *Borrelia* species. In contrast, Borrelial plasmid compositions can vary markedly among even isolates of the same species. Events associated with this variability include plasmid loss and intragenomic recombination (98, 99, 142). The latter may occur through homologous or non-homologous recombination events that result in gene duplications and truncations, as evidenced by the numerous paralogous genes and pseudogenes that have been identified on some circular and most linear plasmids (29, 31, 60). Also unusual for bacterial extrachromosomal elements, Borrelial plasmids (including the prophage cp32 family) encode a variety of primarily lipoprotein factors essential for infectivity and transmission (160). Cp26 carries diverse genes including *resT*, *guaA*, *guaB*, and *ospC* (33, 100) and is uniquely critical to spirochetal survival and *in vitro* growth.

Examinations of chromosomal sequences have also revealed that *Borrelia* retain only basic metabolic capabilities, lacking genes for *de novo* synthesis of amino acids, fatty acids, and nucleotides (60). As a result of their minimalist genomes, *Borrelia* spirochetes are fastidious and entirely dependent upon vertebrate or invertebrate hosts (the genus contains no known free-living

species). Implications of this in the laboratory context include the requirement for highly complex media during cultivation (14).

Pathobiology and immune evasion

RF spirochetes are predominantly blood-borne pathogens and reach densities in the host bloodstream as high as 10^8 spirochetes per milliliter (54, 154). Within the blood, interactions with cellular components may contribute to spirochetal pathogenicity. *B. crocidurae*, *B. duttonii*, and *B. hispanica* have been demonstrated to aggregate erythrocytes into spirocentric rosettes via interaction with erythrocyte neolacto- glycans (70). The role of this process during infection is unknown, but both immune-evasive (26) and disseminative (152) functions have been proposed. Additionally, thrombocytopenia is the most common clinical finding of TBRF (47), and the interaction of *Borrelia* spirochetes with human and rodent platelets has been well-documented (2, 4, 35, 64). In relapsing fever, formation of spirochete-platelet complexes appears to directly induce thrombocytopenia through elevated platelet clearance (4). The precise mechanism of this interaction is unknown, as platelet integrin $\alpha_{IIb}\beta_3$ mediates Borrelial attachment *in vitro* (2, 35) but has been demonstrated to be nonessential during infection (4).

Complications associated with relapsing fever, including neuroborreliosis, arthritis, and carditis, reflect the pathogen's ability to disseminate extravascularly (27, 65, 94, 117, 151). Studies have suggested that, like the closely-related Lyme disease *Borrelia*, relapsing fever spirochetes display tropism for brain and collagenous tissues (28), and that specific tissue tropism may be serotype-dependent (94, 109, 150). In addition to causing direct tissue damage, low-level extravascular persistence may contribute to disease reactivation following immune system compromise (90) or co-infection (e.g., with malaria) (96).

Dissemination from circulating blood involves passage across tissue barriers such as the extracellular matrix and the vascular basement membrane. Recruitment of host-derived plasminogen (zymogen of the serine protease plasmin) has been demonstrated in both Lyme and relapsing fever *Borrelia* (63, 69, 81, 88, 134, 141). In *Borrelia burgdorferi*, plasminogen binding enhances penetration of endothelial monolayers *in vitro* (38, 68), while, for RF spirochetes, plasminogen has been shown to play an important role in heart, kidney, and brain invasion (65, 117).

Host clearance of RF spirochetes is achieved through an IgM-mediated, T cell-independent immune response in which the B1b B cell subclass appears to play a central role (3, 11, 40, 115). This adaptive immune response, although efficacious and rapid, is serotype-specific. Therefore, to evade antibody-mediated immunity, RF *Borrelia* employ a prototypical antigenic variation system involving variable major lipoproteins Vsp and Vlp (9, 15, 161). This system is founded on multiple silent ("archived") versions of *vlp* and *vsp* genes scattered throughout genomic linear plasmids; 59 *vlp*/*vsp* alleles have been identified (43). A single expression locus for these genes resides near the telomere of a 28-kb linear plasmid, into which any one of the silent genes may be copied, in its entirety, through non-reciprocal recombination. Specific upstream and downstream homology sequences appear to mediate this recombination and to dictate an observed hierarchy of switch rates between variants (12). Serotypic variants spontaneously arise, both *in vivo* and in culture, at a frequency of 10^{-4}-10^{-3} events per cell per generation (119).

Innate immunity appears to play a role in controlling Borrelial infections (20), although the mechanisms remain poorly defined. The ability of RF *Borrelia* to functionally evade innate immunity, however, is clearly demonstrated by the massive and sustained bacteremia caused by

these organisms. Many pathogens exploit host complement regulatory processes as a means of innate immune evasion, usually through production and/or recruitment of factors that negatively regulate the complement cascade (6, 18, 19, 61, 74, 112, 113, 118, 123, 125, 128, 140, 165). These mechanisms have been documented in many pathogenic spirochetes including *Leptospira* species (8, 159), Treponemes (103, 104), and Lyme and relapsing fever *Borrelia*.

Complement and factor H

The complement system is comprised of more than 30 soluble and cell-surface proteins that act in concert to eliminate pathogens by opsonization and lytic events. Many complement proteins exist in the body as zymogens that are sequentially activated through a cascade of cleavage reactions. Such activation can occur by way of three pathways (classical, lectin, and alternative) that differ in their mechanisms of initiation but ultimately converge in the terminal stages of activity (72, 138).

Both the classical and the lectin pathway are activated by distinct recognition mechanisms including, for the former, antigen-complexed antibodies, and, for the latter, specific cell-surface arrangements of mannose residues. In contrast, low-level activation of the alternative pathway occurs independently and spontaneously in the plasma. This process results in local pools of activated C3b that bind carbohydrates and proteins on nearby cell surfaces via exposed, highly reactive thioester bonds (120). If binding fails to occur, C3b is quickly inactivated by hydrolysis (172). Upon binding to an activating surface (e.g., a pathogen), C3b is bound by zymogen factor B, which is itself cleaved and activated by factor D. The large Bb fragment remains in association with C3b, forming the alternative pathway C3 convertase, C3bBb. Both the classical and the lectin pathway lead to the generation of functional equivalents of this

enzyme; therefore, the pathways converge at this stage. The C3 convertase generates high local levels of active C3b fragments, which act independently as opsonins or complex with the C3 convertase to form the C5 convertase (for the alternative pathway, $C3b_2Bb$) (173). This enzyme cleaves and activates C5, generating C5a, a potent anaphylatoxin, and C5b, the earliest membrane attack complex (MAC) component. During MAC formation, the terminal complement components assemble as follows: C5b binds C6 and C7 which, in complex, undergo conformational changes and insert into the pathogen cell membrane via a hydrophobic region on C7. This allows C8 to bind, insert into the membrane, and induce the final component, C9, to bind and polymerize. The resulting pore-like structure is cytolytic and promotes elimination of target cells (138).

The complement cascade is tightly regulated by a variety of cell-surface and fluid-phase proteins to prevent uncontrolled or inappropriate activation within the host (175). Factor H (FH) is the major fluid-phase regulator of the alternative complement pathway. A plasma protein of 155 kDa, human FH is composed of individually folding domains known as short consensus repeats (SCR) which are joined by short, flexible linking sequences (139). Domain conformation is maintained by disulfide bonds that form in a 1-3 and 2-4 manner between four conserved cysteine residues harbored within each SCR. A critical element of alternative pathway regulation involves specific discrimination between self-cells and pathogens targeted for destruction. This specificity is largely conferred by FH, which contains multiple heparin-binding sites that interact with polyanionic regions present on human cells and tissues (but generally absent from microbial surfaces), such as glycosaminoglycans (GAGs) and sialic acid residues. The most well-characterized heparin-binding sites are contained within SCRs 7 and 20, and an additional site is present within SCRs 9-15 (57, 139, 140, 176). Interactions with such polyanionic surfaces

enhance the binding affinity of FH for C3b (111) and allow FH to inhibit cascade progression by several mechanisms, including cofactor activity in the factor-I mediated cleavage of C3 and destabilization of the C3 convertase (172).

Borrelial factor H binding and FhbA

As discussed, many pathogenic spirochetes exploit the protective capacity of exogenous FH by recruiting it to surface-expressed proteins during host infection. This process has been most extensively examined in the LD species *B. burgdorferi*, which produces five factor H-binding proteins (FHBPs) including CspA (BBA68/CRASP-1); CspZ (BBH06/CRASP-2); and OspE family proteins BBN38, BBL39, and BBP38 (CRASP3-5, respectively) (1, 23, 73, 80, 102, 107). Other Borrelial species that produce FHBPs include LD spirochete *B. afzelli* and RF spirochetes *B. recurrentis*, *B. parkeri*, and *B. hermsii* (69, 78, 108, 141). Among the RF species, the FH-binding mechanism of *B. hermsii* and its single FHBP, factor H-binding protein A (FhbA), are most well characterized.

FhbA is a ~20-kDa outer surface lipoprotein that is encoded by a single copy gene borne on the 200-kb linear plasmid lp200 (78). Production of FhbA is widespread among low-passage B. *hermsii* isolates; however, several high-passage strains (REN_{HP}, FRO_{HP}, CON_{HP}) have been found to lack the *fhbA* gene and lp200, and rather to harbor a 170-kb linear plasmid. This change is consistent with the linear plasmid rearrangement frequently observed in *Borrelia*, and also suggestive that *fhbA* may be of prophage origin. Two phyletic *fhbA* groups (*fhbA1*, *fhbA2*) have been identified (79) and correlate well with *B. hermsii* genomic group (GG) designations previously established through sequence analyses of chromosomal loci (*flab*, *glpQ*, and *gyrB*) (122). FhbA is expressed during infection and has been demonstrated to be highly immunogenic

(39, 76, 78, 79). In addition to FH, FhbA binds other serum proteins including FHL-1 (a FH splice variant with complement regulatory and adhesive functions) and plasminogen (77).

FH binding is essential to complement resistance in the spirochete *Treponema denticola* (101). In the *Borrelia*, evidence supports the notion that FH-binding ability is linked to resistance to complement-mediated killing *in vitro* and during infection (23, 76, 86, 108, 131). However, direct demonstration of the role of FH binding in the Borrelial disease process, achieved traditionally through gene inactivation, has not yet been possible.

Genetic manipulation of relapsing fever spirochetes

The first demonstration of genetic manipulation of a *Borrelia* species was in the Lyme disease spirochete *B. burgdorferi* (136). Although applications of Borrelial molecular genetics have broadened considerably, the same basic methods are still largely observed (132). While standard techniques are employed to introduce DNA by electroporation, large amounts of DNA are required to overcome the *Borrelia*'s notoriously low transformation efficiency (166). An important and confounding factor in the genetic manipulation of *B. burgdorferi* is transformation-associated loss of endogenous plasmids that encode proteins required for survival in either tick or mammalian hosts (91, 124, 132).

To date, there is only a single report of site-directed allelic exchange in a RF *Borrelia* species (17) and diverse tools for this purpose are lacking. Although chromosome sequences of *B. hermsii* (GGI DAH isolate), *B. turicatae*, *B. recurrentis*, and *B. duttonii* (92) are now available, assembled, annotated, and complete sequences for plasmid components of these genomes have not yet been determined.

Research objectives

Prior to clearance by serotype-specific, antibody-mediated adaptive immune processes, RF spirochetes persist at extremely high levels in the host bloodstream (154). This high-level bacteremia suggests facilitation by spirochetal mechanisms of innate immune evasion. One such putative mechanism involves recruitment of host-derived factors that negatively regulate the complement cascade. Binding of FH, the primary fluid-phase regulator of the alternative complement pathway, has been demonstrated in a variety of pathogens including *Borrelia* species (61, 74, 112, 113, 140). TBRF spirochete *B. hermsii* binds FH via its FhbA protein (78). However, direct demonstration of the role of FhbA in the disease process has been precluded by the lack of a reliable genetic manipulation system for RF *Borrelia*. In an effort to develop molecular tools for this purpose while exploring the role of FH binding in Borrelial virulence, this work addresses the following aims:

1. **Expand the genetic tools available for studying the molecular pathogenesis of RF spirochetes**

   Molecular characterization of TBRF spirochete *B. hermsii* has been hindered by methodological challenges; this objective will involve refining existing techniques to overcome these challenges. Site-specific allelic exchange mutagenesis and a unique kanamycin resistance-green fluorescent protein construct will be utilized to achieve genetic transformation of *B. hermsii* strain YOR. Resulting transformant clones will be characterized both *in vivo* and *in vitro*.

2. **Directly assess the role of FH binding in the virulence of TBRF *Borrelia***

Using genetic transformation techniques, a *B. hermsii* strain YOR *fhbA* deletion mutant will be generated. Analyses of the interaction of this strain with complement proteins will clarify the importance of FhbA expression in FH binding and *in vitro* complement inactivation. Infection and serum sensitivity assays will be used to assess the role of the FH-binding phenotype in innate immune evasion by TBRF spirochetes.

Materials and Methods

Bacterial strains and cultivation

Borrelia hermsii strain YOR was isolated in 1964 from a human relapsing fever patient in Siskiyou County, California (122). *Borrelia andersonii* isolate MOD-1 was originally isolated from nymphal *Ixodes dentatus* ticks in Bollinger County, Missouri (93) (Table 1). *B. hermsii* and *B. andersonii* were cultivated in BSK-H complete media supplemented with 12% and 6% rabbit serum, respectively, at 37°C in a humidified atmosphere with 5% CO_2. Antibiotic selection of transformant strains was achieved with kanamycin (200 μg ml^{-1}).

Isolation of genomic DNA

Cells from log-phase cultures of wild type *B. hermsii* strain YOR were collected by centrifugation (14,000 x *g*, 15 min, 4°C) and washed twice with phosphate buffered saline (PBS) (1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 20 mM KH_2PO_4, pH 7.4). Cells were then resuspended in TES (50 mM Tris-HCl, pH 8.0; 40 mM EDTA; 25% sucrose) and lysed through incubation with SDS (2%) and Proteinase K (0.3 mg ml^{-1}) (37°C, 45 minutes). Liquid-liquid extraction of DNA was performed using phenol/chloroform/isoamyl alcohol (25:24:1) and centrifugal phase separation. Upon aqueous phase recovery, DNA was precipitated by the addition of 3 volumes of cold 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) (30 minutes, -70°C). DNA was collected by centrifugation (14,000 x *g*, 5 min, 4°C), washed twice with 70% ethanol, and allowed to air dry at room temperature. DNA was then incubated in TE

Table 1. Summary of strains used in this study

Strain or Isolate	Source	Comments
B. hermsii YOR (*Bh* YOR-wt)	Human, Siskiyou Co., CA (122)	Wild type strain
Bh YOR::*kan*^{r}*gfp*	This study	*B. hermsii* YOR transformant; bears *kan*^{r}*gfp* insertion
Bh YORΔ*fhbA*	This study	*B. hermsii* YOR transformant; bears *kan*^{r}*gfp* insertion and *fhbA* gene deletion
B. andersonii MOD-1	*Ixodes dentatus* tick, Bollinger Co., MO (93)	Wild type isolate, Non-FH-binding (Marconi, R.T., unpublished data)

(10 mM Tris-HCl, pH 8.0; 1 mM EDTA) with RNase (Abgene; 0.03 mg ml^{-1}), 1% SDS, and Proteinase K (0.3 mg ml^{-1}) for 1 hour at 37°C; extracted and precipitated once again, as above; and resuspended in water. Quantification was achieved by spectrophotometric measurement of UV absorbance (A_{260}/A_{280}).

Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using an S1000 Thermal Cycler (Bio-Rad) and template sources that included genomic DNA, plasmid DNA, and bacterial cells. Preparation of cellular template consisted of collecting and boiling *Borrelia* cells in water (98°C, 12 minutes), and, following brief centrifugation to remove cellular debris, utilization of supernatants at 1 µl $reaction^{-1}$. Genomic and plasmid DNA templates were used at 5.0 and 0.5 ng $reaction^{-1}$, respectively. The final volume of each PCR reaction was 20 µl, and primers (Table 2) were used at constant 15-µM concentrations. For vector construction, Phusion High-Fidelity Master Mix (Phusion High-Fidelity DNA polymerase (0.02 U μl^{-1}), 200 µm each dNTP, 1.5 mM $MgCl_2$; New England BioLabs) was used. Phusion-specific cycling conditions were as follows: 98°C, 30 seconds; (98°C, 10 seconds; 60°C, 10 seconds; 72°C, 30 seconds-1.5 minutes) x 35 cycles; 72°C, 5 minutes. All other PCR applications were performed using GoTaq Green Master Mix (GoTaq DNA polymerase, 200 µm each dNTP, 1.5 mM $MgCl_2$; Promega) and the following cycling conditions: 94°C, 2 minutes; (94°C, 30 seconds; 53-63.5°C, 30 seconds; 72°C, 30 seconds-2 minutes) x 35 cycles; 72°C, 5 minutes. Amplicons were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Vector construction

A suicide vector, designated pFAEV3, was designed to allow stable integration of a kanamycin resistance (*kan*^{r}) cassette and *gfp* into lp200. The integration site consists of a 209-bp non-coding region residing between the 3' end of *fhbA* and the 3' end of an oppositely-oriented hypothetical open reading frame (labeled as ORF dn in Figure 1). Regions of lp200 and *gfp* and *kan*^{r} genes were PCR-amplified from *B. hermsii* YOR genomic DNA and previously constructed plasmids as described below. Primers were tailed with appropriate restriction sites as needed to allow for subsequent cloning procedures (Table 2). First, a 1582-bp region of lp200 was amplified from genomic DNA using the VC1 and VC2 primers. This amplicon extends from upstream of *fhbA* through its stop codon (encompassing the *fhbA* gene and an upstream hypothetical ORF; VC2 harbors an NheI site). The 209-bp non-coding region targeted for insertion was amplified from genomic DNA in two fragments: (i) the 99-bp region immediately 3' of *fhbA*, using VC3 and VC4 primers (with NheI and SalI restriction sites, respectively), and (ii) a 625-bp region encompassing the remaining non-coding sequence and a downstream ORF of unknown function, using VC9 and VC10 primers (with AatII and MluI restriction sites, respectively). The *kan*^{r} gene, linked to the constitutively active *B. hermsii* DAH *flgB* promoter (*PflgB-kan*^{r}*)*, was amplified from pTABhFlgB-Kan (17) (kindly provided by Dr. T. Schwan) using the VC5 and VC6 primers (with SalI and AscI restriction sites, respectively). *gfp* was amplified from pCE320 (51) (kindly provided by Dr. J. Radolf) using the VC7 and VC8 primers (with AscI and AatII sites, respectively). The *kan*^{r} and *gfp* genes were linked to allow for expression from the *kan*^{r} cassette. The two genes are separated by 60 bp of sequence derived from the region upstream of the *gfp* gene. All amplicons were cut with the appropriate restriction enzymes (New England Biolabs) and, using standard subcloning techniques, ligated into a pCR2.1 TOPO backbone (Invitrogen) to yield pFAEV3 (Figure 1A).

Generation of a *B. hermsii* YOR *fhbA* deletion mutant was achieved through allelic exchange mutagenesis using suicide vector construct pFAEV3Δ*fhbA*. This vector was derived from pFAEV3, described above. To construct pFAEV3Δ*fhbA*, the pFAEV3 vector was subjected to digestion with NheI, resulting in excision of the coding region of *fhbA* from the vector sequence. The construct was then re-ligated to yield the final pFAEV3Δ*fhbA* vector (Figure 1B).

Transformation of *B. hermsii*

Plasmids were propagated in NovaBlue *Escherichia coli* cells (Novagen), purified (HiSpeed Plasmid Midi Kit; Qiagen) according to manufacturer's instructions, and linearized with AhdI and PflfI prior to transformation. Electroporation conditions were adapted from methods originally developed by Samuels et al. for *B. burgdorferi* (136). *B. hermsii* YOR cells were harvested from a 100-ml, mid-log phase culture by centrifugation (5000 x *g*, 20 min, 4°C), washed 3 times with cold EPS buffer (93 mg ml^{-1} sucrose, 15% glycerol), and suspended in 100 µl EPS buffer containing 25 µg of linearized pFAEV3 or pFAEV3Δ*fhbA* vector. After 5 min on ice, the cells were electroporated (0.2 cm cuvette, 2.5 kV, 25 mF, 200 W), transferred to 10 ml of BSK-H media with 12% rabbit serum, and incubated for 24 hours at 37°C. The culture volumes were increased to 50 ml with fresh BSK-H medium and supplemented with kanamycin (200 µg ml^{-1}). Cultures were maintained at 37°C and growth was monitored by darkfield microscopy.

Transformant clones were isolated through limiting dilution. Sequential dilutions containing, theoretically, 50 to 0.00064 spirochetes were prepared and cultured in sterile 96-well tissue culture plates (Becton Dickinson) containing 200 µl BSK-H media with 200 µg ml^{-1} kanamycin per well. These plates were then sealed with adhesive plate covers (EdgeBio) and incubated at 37°C (5% CO_2). Growth was monitored by darkfield microscopy.

Figure 1. *B. hermsii* pFAEV3 and pFAEV3Δ*fhbA* suicide vector maps. Suicide vector pFAEV3 was constructed to allow for the introduction of a selectable marker (*kan*^{r}) and green fluorescent protein (*gfp*) into a non-coding region of the native *B. hermsii* linear plasmid, lp200. The vector map is depicted in Panel A. The primers used to generate the amplicons for vector construction (VC) are indicated (listed in Table 2). (B) *B. hermsii fhbA* deletion vector (pFAEV3Δ*fhbA*) was derived from pFAEV3, through NheI digestion, to permit *kan*^{r}*gfp* cassette integration and deletion of the *fhbA* coding region from lp200.

Figure 1

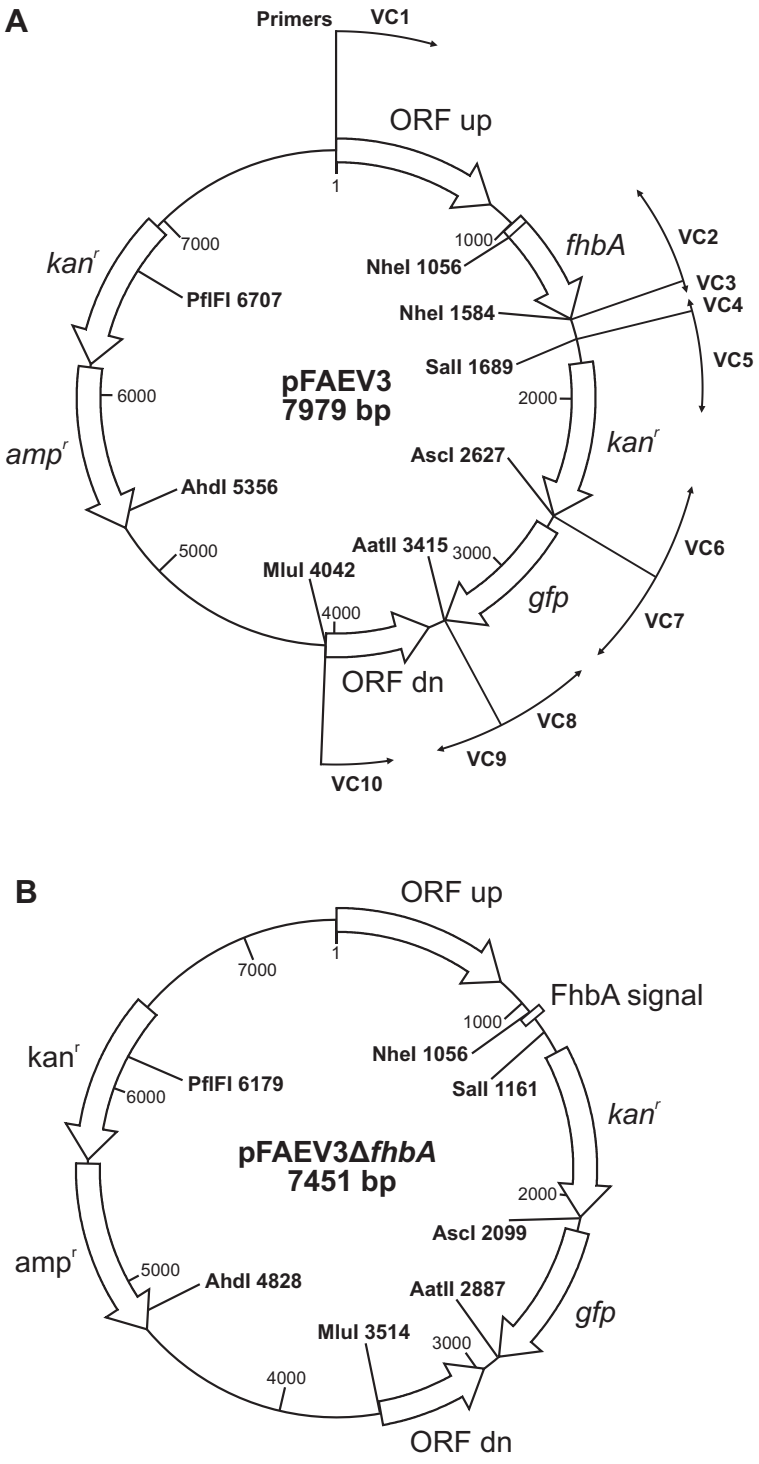

A
Primers
VC1
ORF up
1
1000
fhbA
VC2
VC3
VC4
VC5
Nhel 1056
Nhel 1584
Sall 1689
2000
kanr
Ascl 2627
7000
kanr
PflFI 6707
6000
pFAEV3
7979 bp
ampr
Ahdl 5356
5000
AatII 3415
3000
VC6
MluI 4042
gfp
4000
VC7
ORF dn
VC8
VC9
VC10
B
ORF up
1
7000
FhbA signal
1000
kanr
Nhel 1056
PflFI 6179
6000
Sall 1161
kanr
pFAEV3ΔfhbA
7451 bp
2000
Ascl 2099
ampr
5000
Ahdl 4828
AatII 2887
gfp
MluI 3514
3000
4000
ORF dn

Table 2. Oligonucleotide primers

Primer	Sequence (5' to 3')
VC1	GTCAATGGTATATTAGTTTCAAGTTCTATGACCC
VC2	ACGCGTAAAGTCGACTTAACATATAAATAATACTGATATTAAAATTTATGTAAA
VC3	GCTAGCGTTGATCTTATAAATCAAACTGATGTAAAATCAAAACC
VC4	GTCGACTTAACATATAAATAATACTGATATTAAAATTTATGTAAA
VC5	GTCGACGTTAAAGAAAATTGAAATAAACTTGGACTATGTTAATG
VC6	GGCGCGCCTTAGAAAAACTCATCGAGCATCAAATG
VC7	GGCGCGCCGGGCGAATTCGGCTTATTCC
VC8	GACGTCCTATTTGTATAGTTCATCCATGCCATGTG
VC9	GACGTCCCTGAGAGTATAGGCAGTACTTCAATTACTACC
VC10	ACGCGTCAATTGATATTGATGTGGCGTTGATTTCTAAAATAG
TC1	GTCTACTCTATCTGGGATACTAAAGAGC
TC2	TGTGATTTATTCAATAAAAACAAAAAATTAGATGC
TC3	TTAAGTTTTTAAATATTCCATTATAGTTTCAAAAAAATTGTC
TC4	GAGGCATAAATTCCGTCAGC
TC5	GTCCACACAATCTGCCCTTTC
TC6	GCATTAAGTAAGCGGTTTGTGAGC
F1	ATCAGTGAGCTCGATTTATTCAATAAAAACAAAAAATTAGATGCTGATTTAC
F2	TGATACCTCGAGTTAAGTTTTTAAATATTCCATTATAGTTTCAAAAAAATTG

Abbreviations used in primer designations are as follows: VC=vector construction; TC=transformation confirmation. Restriction site/sacrificial base tails are indicated by underlining.

Growth curve analyses

To assess growth rates, equal numbers of actively growing cells of each *B. hermsii* YOR strain were inoculated into fresh medium and maintained at 37°C. Microscopic cell counts of each culture were conducted daily for seven days (from ten fields under 400X magnification, using darkfield microscopy) and the average numbers of spirochetes per field of view were determined at each time point.

Plasmid analysis by pulsed-field gel electrophoresis

Cells from log-phase cultures were collected by centrifugation (6,000 x *g*, 15 min, 4°C), washed twice with PBS, and resuspended in TN buffer (50 mM Tris-HCl, pH 8.0; 150 mM NaCl) to a density of ~1 x 10^9 cells ml^{-1}. Cell suspensions were warmed to 37°C then mixed with an equal volume of 1.2% agarose (InCert; FMC). Agarose-cell mixtures were transferred to plug casters and allowed to solidify at room temperature. To lyse embedded cells, plugs were sequentially incubated in PK solution (50 mM Tris-HCl, pH 8.0; 50 mM EDTA; 1.5% SDS; 1 mg ml^{-1} Proteinase K) at 45°C, and TE* wash (10 mM Tris-HCl, pH 8.0; 10 mM EDTA) at 50°C. Plugs were then loaded in 1% agarose gels (SeaKem Gold; Lonza) prepared with 0.5x TBE buffer (44.5 mM Tris base; 44.5 mM boric acid; 1 mM EDTA, pH 8.0). Electrophoresis was performed in 0.5x TBE buffer at 14°C using a contour-clamped homogeneous electric field (CHEF) mapper system (Bio-Rad). Automatic parameters for separation of DNA molecules between 5 and 200 kb were utilized (calibration factor, 1.00; gradient, 6.0 V/cm; run time, 15:16; included angle, 120°; initial switch time, 0.22 s; final switch time, 17.33 s; ramping factor, a=linear). DNA was visualized by ethidium bromide staining.

Immunoblot analyses

B. hermsii cells were solubilized in reducing sample buffer (0.25 M Tris-HCl, pH 8.0; 4% SDS; 2% β-mercaptoethanol; 20% glycerol; 0.001% bromophenol blue**),** fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (200 V, 60 minutes) in 15% Criterion Tris-HCl precast gels (Bio-Rad), and transferred to polyvinylidene diflouride (PVDF) by electroblotting (100V, 60 minutes). Immunoblots were blocked in blocking buffer (PBS, 0.2% Tween 20, 5% nonfat dry milk) for 1 hour, then incubated with primary antibody. Primary antibodies included mouse anti-FhbA antiserum (1:1000) and mouse anti-FlaB antiserum (1:400,000), diluted in blocking buffer as indicated. Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (1:40,000; Pierce) served as the secondary antibody. Incubations were performed at room temperature for 1 hour, and were followed each time by PBS-0.2% Tween 20 (PBS-T) wash steps to remove unbound antibody. Detection was based on the reaction of the HRP conjugate with SuperSignal West Pico chemiluminescent substrate (Pierce).

Proteinase K digestion of surface proteins

Cells (~3.5 x 10^8) from log-phase *B. hermsii* cultures were collected by centrifugation (5,000 x *g*, 20 min, 4°C), washed with PBS, and resuspended in PBS with or without Proteinase K (0.2 mg ml^{-1}). After 1 hour at room temperature, digestion was inhibited by the addition of phenylmethanesulfonylfluoride (PMSF) (5 ug ml^{-1}). Cells were collected by centrifugation (14,000 x *g*, 10 min, room temperature) and subjected to immunoblot analysis, as described above, to assess FhbA expression at the cell surface. Blots were also screened with anti-FlaB (a

constitutively expressed flagellar protein) to demonstrate specific digestion of surface-exposed proteins and to confirm equal sample loading.

Affinity ligand binding immunoblot (ALBI) assays

B. hermsii cell lysates were fractionated by SDS-PAGE under reducing conditions and transferred to PVDF as described above. After blocking, immunoblots were incubated with purified human FH (5 ng μl^{-1}; CompTech) in blocking buffer for 1 hour at room temperature, then washed with PBS-T. Bound FH was detected with goat anti-human FH antiserum (1:1000; Calbiochem) followed by HRP-conjugated rabbit anti-goat IgG (1:40,000; Pierce). Incubations were performed in blocking buffer at room temperature for 1 hour, and blots were washed between incubation steps. Detection was based on chemiluminescence.

Whole-cell adsorption of FH and IFA

To assess binding of FH by intact spirochetes, whole-cell FH adsorption assays were performed. Cells were harvested from 5-ml, mid-log phase cultures by centrifugation (5000 x *g*, 10 min, 4°C), washed with cold PBS, and resuspended in 100 µl PBS (supplemented with 1 mM MgCl and 0.15 mM CaCl). The cell suspensions were then incubated with purified human FH (0.525 mg ml^{-1}) for 1 hour at room temperature. Following extensive washing with PBS, cells were recovered by centrifugation (3000 x *g*, 10 min, room temperature) and FH adsorption was assessed by both immunoblot analysis and immunofluorescence assay (IFA). Immunoblot analyses were performed as described above: First, cells were solubilized in reducing sample buffer, fractionated by SDS-PAGE, and transferred to PVDF. The immunoblots were then blocked and screened with goat anti-human FH antiserum (1:1000) followed by HRP-conjugated

rabbit anti-goat IgG (1:40,000). Washing was performed between each step, and detection was achieved through chemiluminescence. For IFA, cells, adjusted to a density of ~7.0 x 10^6 ml^{-1} in PBS, were spotted onto slides (Superfrost Plus; Fisher) and allowed to dry. The slide-immobilized cells were blocked with 3% bovine serum albumin (BSA) in PBS-T, then probed with goat anti-human FH antiserum at a dilution of 1:5000. The slides were washed three times with PBS-T, then incubated with Alexa Fluor 568-conjugated rabbit anti-goat IgG (1:200; Invitrogen). All antisera were diluted in PBS-T with 3% BSA, and incubations were performed in darkened, humidified chambers for 1 hour at room temperature. After a final wash step, cover slips were mounted using an antifade reagent (ProLong Gold; Invitrogen), and the immobilized cells were visualized by fluorescence microscopy.

Plasminogen binding assay and ELISA

Cells from actively growing cultures of each *B. hermsii* strain were collected by centrifugation (5000 x *g*, 15 min, 4°C) and washed with PBS. Cells were suspended in carbonate coating buffer (27.6 mM sodium carbonate, 18.9 mM sodium bicarbonate, pH 9.6) to an adjusted concentration of ~1 x10^8 cells ml^{-1}, then incubated in 96-well ELISA plates (Costar 3590; Corning) at 100 µl $well^{-1}$ overnight at 4°C. Plate-immobilized cells were blocked with 5% BSA in PBS-T, then incubated with plasminogen (10 µg ml^{-1} in 5% BSA-PBS-T; Sigma) for 1 hour at room temperature. Control wells were (i) coated with wild type *B. hermsii* YOR cells and incubated with 5% BSA-PBS-T only, and (ii) left uncoated, then blocked and incubated with plasminogen. To remove unbound plasminogen, plates were washed three times with PBS-T using an automated EL_x405 Microplate Washer (Bio-Tek). To assess plasminogen binding by enzyme-linked immunosorbent assay (ELISA), plates were screened with goat anti-plasminogen

antiserum (1:1000) and then washed as above. Bound primary antibodies were detected with HRP-conjugated rabbit anti-goat IgG (1:40,000) and 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) chromogenic substrate. All incubations were performed in 5% BSA-PBS-T for 1 hour at room temperature. Absorbance was read at 405 nm using an ELISA plate reader (EL_x808; Bio-Tek).

C3b cleavage assay

To assess the role of FH binding in C3b inactivation by *B. hermsii*, cells (1×10^8) of each *B. hermsii* strain were harvested from mid-log phase cultures by centrifugation (3000 x *g*, 15 min, 4°C). Cells were washed with cold PBS, suspended in PBS (with 10mM $MgCl_2$) (50 µl), and incubated with or without purified human FH (2 µg ml^{-1}; 37°C; 1 hour). After thorough washing, cells were incubated in PBS (with 10mM $MgCl_2$) (20 µl) with purified factor I (150 ng; Calbiochem) and C3b (250 ng; CompTech) for 2 hours at 37°C. As a control, FH (100 ng), FI (150 ng), and C3b (250 ng) were co-incubated in solution. Following sedimentation of cells, reaction supernatants were mixed with reducing sample buffer, fractionated by SDS-PAGE, and blotted as described above. The presence of C3b cleavage products was assessed by immunoblot analysis using goat anti-human C3 antiserum (1:800; CompTech), HRP-conjugated rabbit anti-goat IgG (1:40,000), and chemiluminescence.

Serum sensitivity assay

Aliquots of log-phase cultures of *B. hermsii* strains and *B. andersonii* isolate MOD-1 (a serum sensitive control) were incubated at 37°C in 50% normal human serum (NHS) (Valley Biomedical) or heat-inactivated (56°C, 30 minutes) human serum (hiNHS). After 2, 6, and 24

hours, cells were stained using the LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen) according to manufacturer's instructions, and examined by fluorescence microscopy (ten fields, 400X magnification) to assess survival. Data are presented as percent survival, calculated as: [number alive/(number alive + number dead)] x 100.

Cell-surface deposition of C9

Cells (1×10^6) from mid-log phase cultures of *B. hermsii* YOR strains and *B. andersonii* isolate MOD-1 were collected by centrifugation (5000 x *g*, 15 min, 4°C), washed with cold PBS, and incubated in 10% NHS or hiNHS for 30 min at 37°C. Following extensive washing, cells were immobilized onto ELISA plates in carbonate coating buffer as described above and screened with goat anti-C9 antiserum (1:1000; CompTech) and HRP-conjugated rabbit anti-goat IgG (1:40,000). Detection was achieved using ABTS chromogenic substrate. Blocking and incubations were performed in 1% BSA-PBS-T. All other ELISA conditions were as described above.

Infection analyses

Infectivity was assessed by subcutaneous needle inoculation of C3H/HeJ mice (n=5) with 5×10^4 spirochetes in BSK-H media. One uninfected mouse of the same cohort was maintained as a control. Blood samples were collected from tail nicks performed 0, 3, 4, 7, 10, 14, 18, and 42 days post-infection and were mixed with sodium citrate buffer (0.15 M trisodium citrate in water) at a 9:1 ratio to prevent clotting. The presence of spirochetes at each time point (with the exception of Day 42) was assessed visually by darkfield microscopy of diluted blood samples (1:20 in PBS; 5 µl). Data are presented as number of spirochetes ml^{-1} blood, calculated using the

following conversion factor: number of spirochetes 400X field^{-1} x (2.5 x 10^{3}) = number of spirochetes ml^{-1}. Additionally, at each time point, undiluted blood (6 μl) was added to BSK-H media (supplemented with rifampicin, fosfomycin, and amphotericin B) and incubated at 37°C. Cultures were microscopically assessed for spirochete growth after 3 weeks of incubation.

Serological analyses and generation of recombinant protein

Blood was collected at 4 weeks post-inoculation and serum ("infection serum") was harvested using standard centrifugal methods. To assess murine seroconversion, ELISA plates were coated with a suspension of wild type *B. hermsii* YOR cells in carbonate buffer, as described above. Plates were blocked with 1% BSA-PBS-T and wells were screened in triplicate with a dilution series (from 1:50 to 1:109,350) of infection serum from each mouse. After washing, bound anti-*Borrelia* IgG was detected using HRP-conjugated goat anti-mouse IgG (1:20,000) and ABTS chromogenic substrate. Incubations were performed in 1% BSA-PBS-T. All other ELISA conditions were as described above.

To determine whether sera of infected mice was reactive against FhbA, recombinant *B. hermsii* YOR FhbA (r-FhbA) was generated, immunoblotted as described above, and screened with infection sera (pooled by experimental group; 1:2500). Detection was achieved using goat anti-mouse IgG (1:40,000) and chemiluminescence. To generate the r-FhbA protein utilized in this assay, the coding sequence of *fhbA* (exclusive of the signal sequence) was PCR-amplified from *B. hermsii* YOR genomic DNA using primers tailed by restriction sites compatible with subsequent cloning steps (F1 and F2; Table 2). Amplicons were gel purified (QIAquick Gel Extraction Kit; Qiagen), digested with SacI and XhoI, and ligated into the pET45b(+) vector (Novagen) through standard methods. The resulting plasmids were propagated according to

manufacturer's instructions in NovaBlue and BL21 (DE3) *E. coli* cells (Novagen). Following IPTG induction, recombinant proteins, bearing 1.7 kDa N-terminal His-Tags, were purified from *E. coli* cell lysates using a His-Bind purification kit (Novagen) as directed by the manufacturer.

Microscopy

All microscopy was performed using an Olympus BX51 microscope fitted with a DP71 camera (Olympus) and fluorescein and rhodamine filter sets. Darkfield and fluorescence micrographs were recorded using DP Controller 3.1.1.267 software (Olympus).

Results

Generation of *B. hermsii* YOR transformants

To generate a *B. hermsii* strain that harbors a selectable marker (*kan*^{r}) and constitutively expresses a fluorescent marker (*gfp*) (*Bh* YOR::*kan*^{r}*gfp*), the pFAEV3 vector was constructed (Figure 1A) and electroporated into *B. hermsii* YOR. Transformation resulted in the insertion of a *kan*^{r}*-gfp* cassette into a non-coding sequence located on lp200 between the wild type *fhbA* gene and a hypothetical ORF (Figure 2A). The *B. hermsii* YOR *fhbA* deletion mutant (*Bh* YORΔ*fhbA*) was generated using the pFAEV3Δ*fhbA* vector (Figure 1B), which mediated deletion of the *fhbA* coding region from lp200 and integration of the *kan*^{r}*-gfp* cassette as described above (Figure 2A). Clonal populations of *Bh* YOR::*kan*^{r}*gfp* and *Bh* YORΔ*fhbA* were derived through limiting dilution.

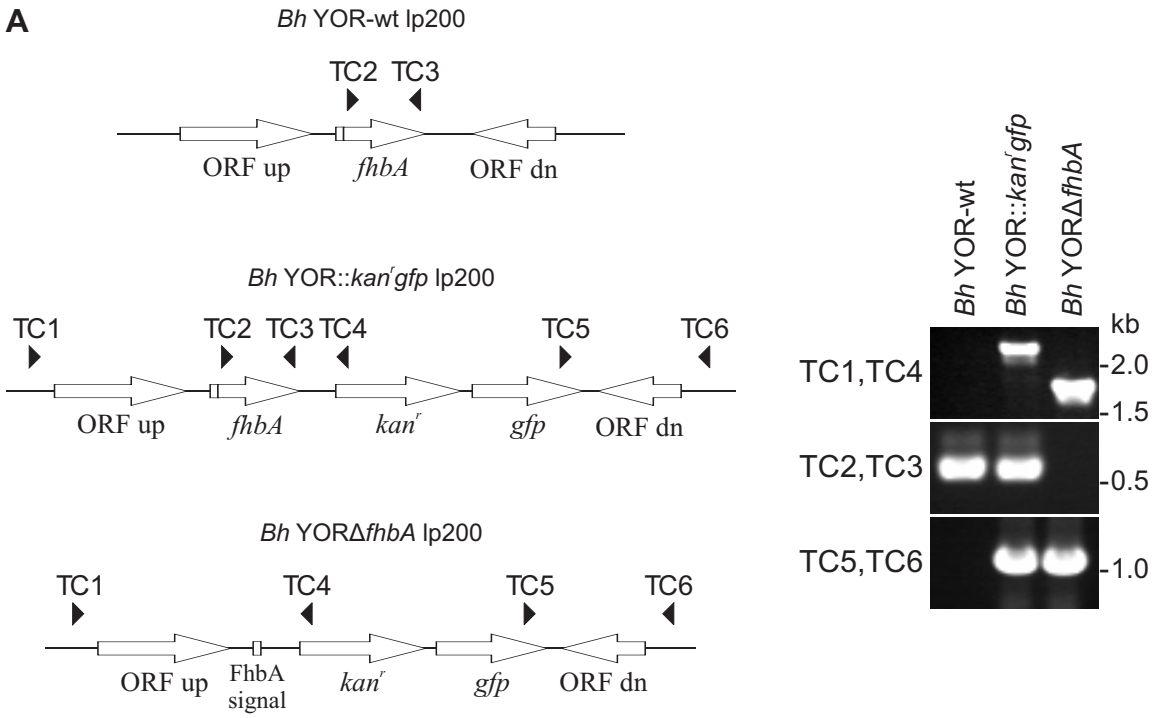
PCR analyses (Figure 2A) and fluorescence microscopy (Figure 2B) were performed to verify successful transformation. All transformant spirochetes observed by fluorescence microscopy produced GFP, although some cell-to-cell variability in signal intensity was observed (data not shown).

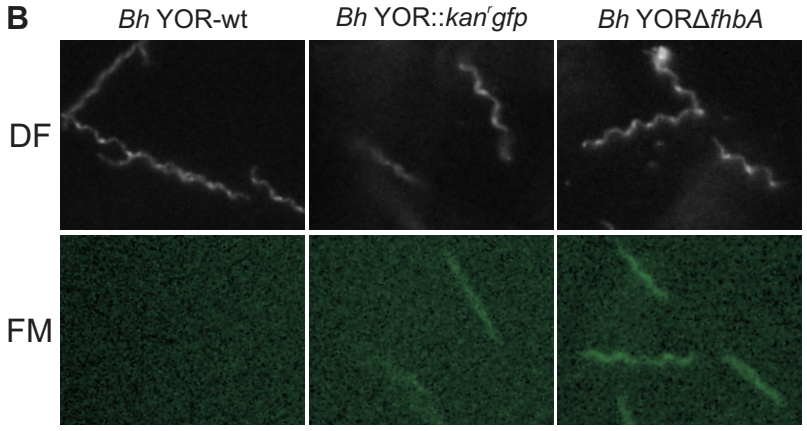
Characterization of plasmid composition and growth kinetics

Electroporation of *B. burgdorferi* often results in the loss of some plasmids (91, 124, 132). Since *Borrelia* plasmids encode genes required for survival in mammalian and arthropod hosts, plasmid loss can influence the interpretation of mutagenesis studies. To determine if

Figure 2. PCR- and microscopy-based verification of allelic exchange. Panel A presents a schematic depicting lp200 from wild type *B. hermsii* YOR (*Bh* YOR-wt) and *B. hermsii* transformant strains (*Bh* YOR::*kan*^{r}*gfp, Bh* YOR∆*fhbA*) after mutagenesis. Locations of primer targets for PCR used to confirm transformation (TC primers) are indicated. The ethidium bromide-stained amplicons obtained through PCR analyses are shown. Primer sequences can be found in Table 2. (B) Fluorescence microscopy (FM) was performed to screen strains for GFP production during *in vitro* culture. Darkfield microscopy (DM) was used to confirm the presence of spirochetes in the selected fields.

Figure 2


A
Bh YOR-wt lp200
TC2 TC3
ORF up
fhbA
ORF dn
Bh YOR::kanrgfp lp200
TC1
TC2 TC3 TC4
TC5
TC6
ORF up
fhbA
kanr
gfp
ORF dn
Bh YORΔfhbA lp200
TC1
TC4
TC5
TC6
ORF up
FhbA signal
kanr
gfp
ORF dn
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA
kb
TC1,TC4
TC2,TC3
TC5,TC6
2.0
1.5
0.5
1.0


B
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA
DF
FM

genetic manipulation of *B. hermsii* YOR resulted in plasmid loss, the plasmid compositions of *Bh* YOR::*kan*^{r}*gfp* and *Bh* YORΔ*fhbA* were assessed by pulsed-field gel electrophoresis (PFGE). This approach revealed the plasmid profiles of the parental and transformant strains to be indistinguishable (Figure 3). Additionally, growth curve analyses demonstrated that *Bh* YOR::*kan*^{r}*gfp* and *Bh* YORΔ*fhbA* grow normally in culture (Figure 4), suggesting that neither insertion of the *kan*^{r}*gfp* cassette nor deletion of *fhbA* interfere with *in vitro* viability.

Analyses of FhbA/factor H-binding protein expression

Expression and cell-surface presentation of FhbA were assessed by immunoblot analyses coupled to Proteinase K digestion. Following incubation either with Proteinase K or with PBS alone, cells of each *B. hermsii* YOR strain were solubilized, fractionated by SDS-PAGE, and probed with anti-FhbA antiserum. *Bh* YOR::*kan*^{r}*gfp* was found to express FhbA after treatment with PBS, but not after digestion with Proteinase K, demonstrating that *kan*^{r}*gfp* cassette insertion does not interfere with cell-surface expression of the adjacent *fhbA* gene (Figure 5A). *Bh* YORΔ*fhbA*, however, failed to express FhbA under control or experimental conditions, suggesting elimination of FhbA expression in this strain (Figure 5A). Factor H affinity ligand binding immunoblot (FH ALBI) analysis was performed to further assess factor H-binding protein expression. Untreated *B. hermsii* cells were solubilized, immunoblotted, and incubated with purified human FH. Proteins capable of retaining FH were detected using anti-FH antiserum. Wild type *B. hermsii* YOR (*Bh* YOR-wt) and *Bh* YOR::*kan*^{r}*gfp* bound FH to an ~20.5 kDa protein presumed to be FhbA, while no *Bh* YORΔ*fhbA* proteins capable of binding FH were identified (Figure 5B). For both assays, identical blots were screened with antiserum against the

Figure 3

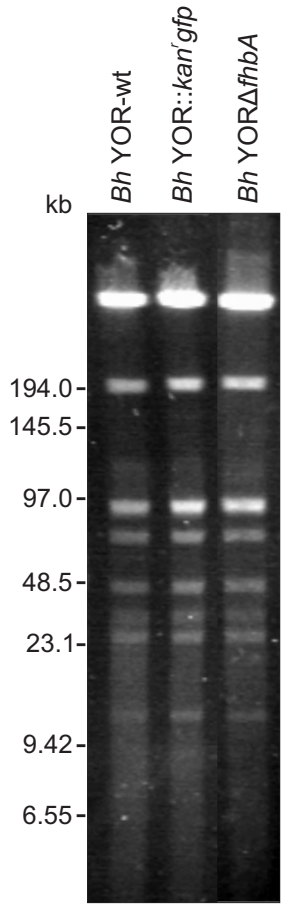


Figure 3. Assessment of plasmid content by PFGE. The plasmid content of each *B. hermsii* YOR strain was assessed using pulsed-field gel electrophoresis (PFGE) and ethidium bromide staining. DNA size standards (in kb) are indicated to the left of the panel.

Figure 4

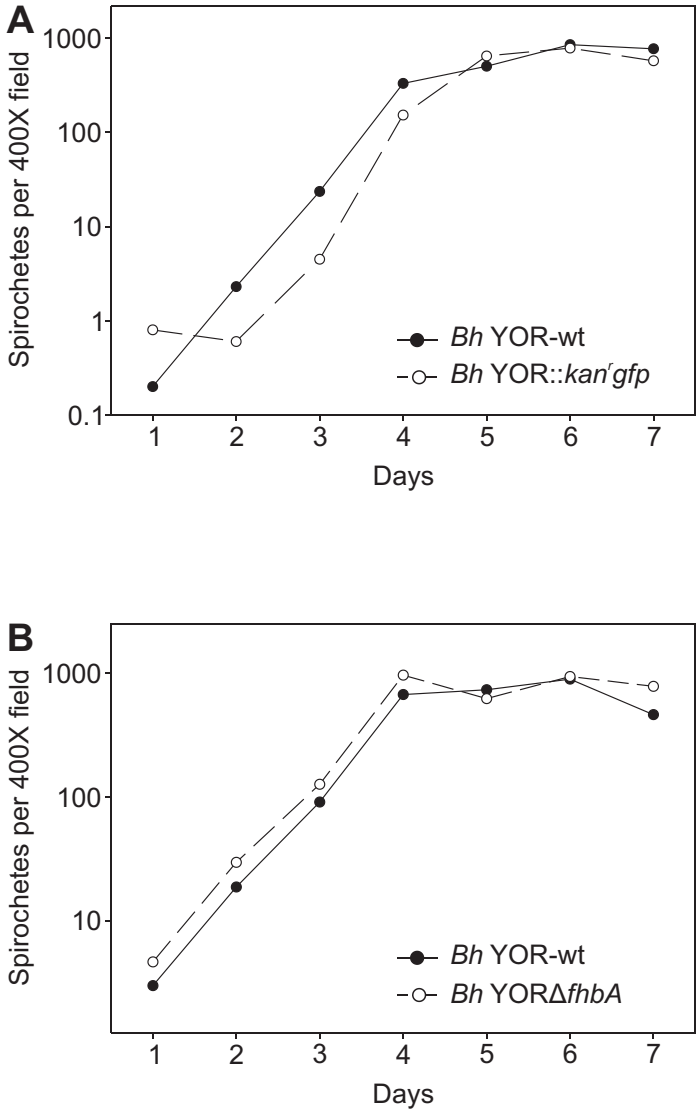


Figure 4. Growth curve analyses. Growth curves for each *B. hermsii* YOR strain were determined through microscopic cell counts (400X magnification) of equivalently inoculated cultures each day for seven days. The growth curves of *Bh* YOR::*kan*^{r}*gfp* and *Bh* YOR*Δfhb*A were compared to the growth curve of the parental wild type strain (*Bh* YOR-wt) in separate experiments, presented in Panel A and Panel B, respectively.

Figure 5

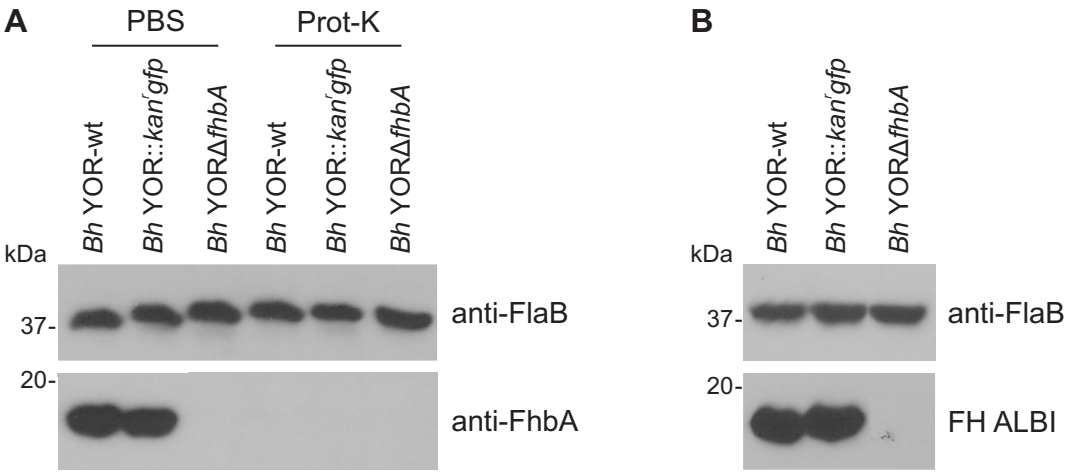


Figure 5. Analyses of FhbA expression by immunoblot and FH ALBI. FhbA expression was assessed (A) by immunoblot analysis of spirochetes that were pre-treated with or without Proteinase K, and (B) by FH ALBI analysis. The ALBI assay was performed by incubating immunoblots of lysates from untreated cells with purified human FH. After washing, FH bound by membrane-immobilized *B. hermsii* proteins was detected using anti-FH antiserum. In both assays, identical blots were screened with anti-FlaB antiserum to confirm equal sample loading. For the Proteinase K assay, this control additionally demonstrated specific digestion of surface-exposed proteins.

periplasmic flagellar protein FlaB to demonstrate equal loading of samples and, for the Proteinase K assay, specific digestion of surface-exposed proteins.

Analysis of the role of FhbA in whole-cell adsorption of FH

Although previous studies (78, 106) suggest that FhbA is the only factor H-binding protein produced by *B. hermsii*, the contribution of FhbA to the factor H-binding phenotype has not been directly assessed. To evaluate this under biologically relevant conditions, we incubated whole spirochetes with purified human FH. After washing, cell surface adsorption of FH was assessed. When screened by immunofluorescence assay (IFA), *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* cells demonstrated cell-surface FH adsorption, while FH was not detected in association with *Bh* YORΔ*fhbA* cells (Figure 6A). Screening of FH-treated cells by immunoblot analysis also showed that *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp*, but not *Bh* YORΔ*fhbA*, adsorb FH from human serum (Figure 6B).

Analysis of C3b inactivation

Wild type *B. hermsii* cells that have been pre-incubated with FH are able to inactivate C3b in a factor I-mediated manner (106). To assess whether this ability is lost with abrogation of FH binding, strains of *B. hermsii* YOR were incubated with or without FH, washed, and then incubated with C3b in the presence of factor I. None of the strains displayed inherent C3b-cleavage ability, and only intact C3b α' and β chains (which migrate separately under reducing conditions) were detected in the reaction mixtures of non-FH-adsorbed cells (Figure 7). When pre-incubated with FH, however, the FhbA-expressing *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* strains degraded C3b. This was evidenced by the accumulation of α'43 and α'68 C3b cleavage products

Figure 6

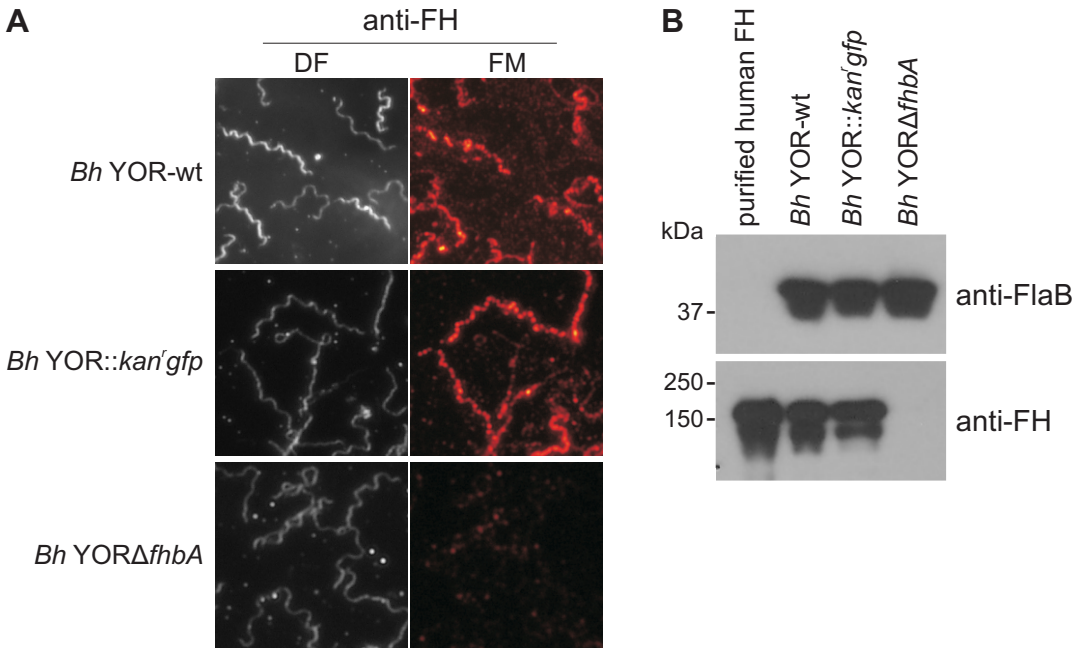


Figure 6. Assessment of factor H binding through whole-cell adsorption assays. Intact cells of each *B. hermsii* YOR strain were incubated with purified human FH. To detect surface-adsorbed FH, the cells were analyzed by (A) immunofluorescence assay (DF=darkfield microscopy, FM=fluorescence microscopy), and (B) immunoblot analysis. Screening for both assays was performed using anti-human FH antiserum and appropriate secondary antibodies. During immunoblot analysis, an identical blot was screened with anti-FlaB antiserum to demonstrate equal loading of samples.

in the respective reaction mixtures (comparable to the cleavage products generated by the purified FH control). In contrast, *Bh* YORΔ*fhbA* failed to cleave C3b regardless of FH pre-incubation (Figure 7).

FhbA expression and the binding of plasminogen by *B. hermsii* YOR

Binding of plasminogen/plasmin by *Borrelia* may play an important role in invasion and dissemination within the host (37, 117). In addition to binding FH, *B. hermsii* FhbA has been shown to bind other host-derived serum proteins including plasminogen (76). The effect of *fhbA* deletion on *B. hermsii* plasminogen-binding ability was assessed by ELISA. Although slightly decreased levels of bound plasminogen were detected for *Bh* YORΔ*fhbA* as compared to *Bh* YOR-wt, all strains bound plasminogen at levels well above background (as established by controls) (Figure 8).

Comparative analyses of serum sensitivity and C9 deposition

To assess the role of FhbA in complement evasion *in vitro*, *B. hermsii* wild type and transformant strains were incubated with either normal, complement-active serum (designated NHS) or heat-inactivated human serum (hiNHS). Serum sensitive *B. andersonii* isolate MOD-1 served as a control. At 2, 6, and 24 hours, the bacteria were examined for viability using *Bac*Light LIVE/DEAD staining. Consistent with previous results (76), *Bh* YOR-wt remained resistant to killing in normal human serum, as did transformant strain *Bh* YOR::*kan*^{r}*gfp* (Figure 9A). In contrast, *B. andersonii* was largely killed within 2 hours, confirming the complement activity of the normal serum. Despite being unable to inactivate C3b through FH/FI-mediated

Figure 7

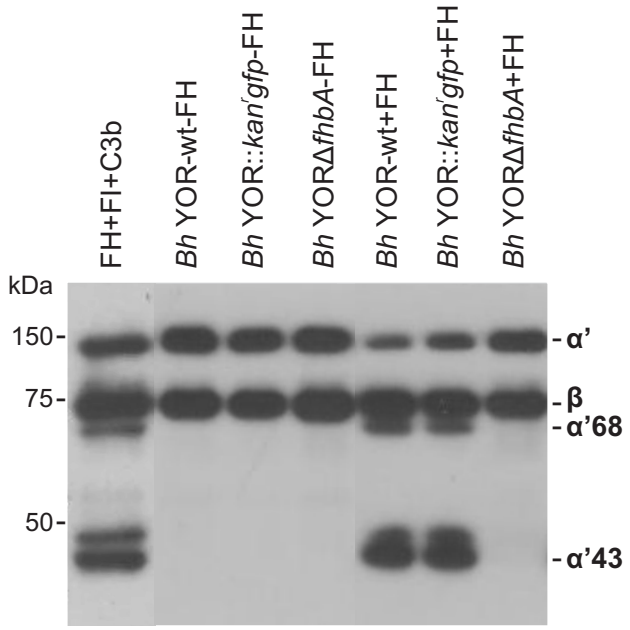


Figure 7. Analysis of C3b cleavage. Cells of each *B. hermsii* YOR strain were pre-treated with or without FH, as indicated, prior to incubation with factor I and C3b. As a control, FH, factor I, and C3b were co-incubated in solution. Following removal of cells, the reaction mixtures were immunoblotted and screened with anti-C3 antiserum. Expected sizes of the C3b α' and β chains, as well as the factor-I mediated C3b cleavage products α'43 and α'68, are indicated to the right of the panel.

Figure 8

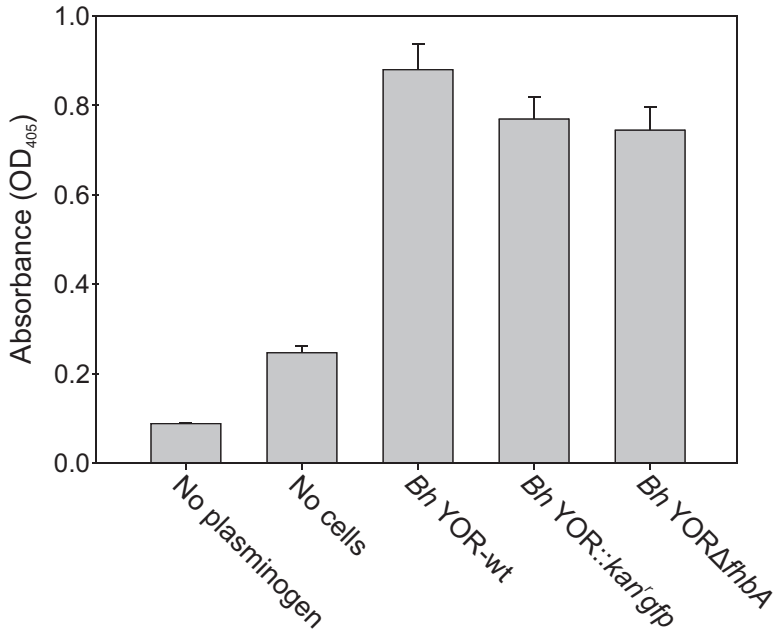


Figure 8. Analysis of the effects of *fhbA* deletion on *B. hermsii* plasminogen binding. Cells of each *B. hermsii* YOR strain were immobilized into 96-well plates, incubated with plasminogen, and screened by ELISA to detect bound plasminogen. Controls consisted of *Bh* YOR-wt cells incubated with BSA alone (No plasminogen) and uncoated wells incubated with plasminogen (No cells). All samples were run in triplicate. Error bars represent standard deviation.

cleavage (Figure 7), *Bh* YORΔ*fhbA* did not display increased serum sensitivity and remained resistant to killing out to 24 hours (Figure 9A).

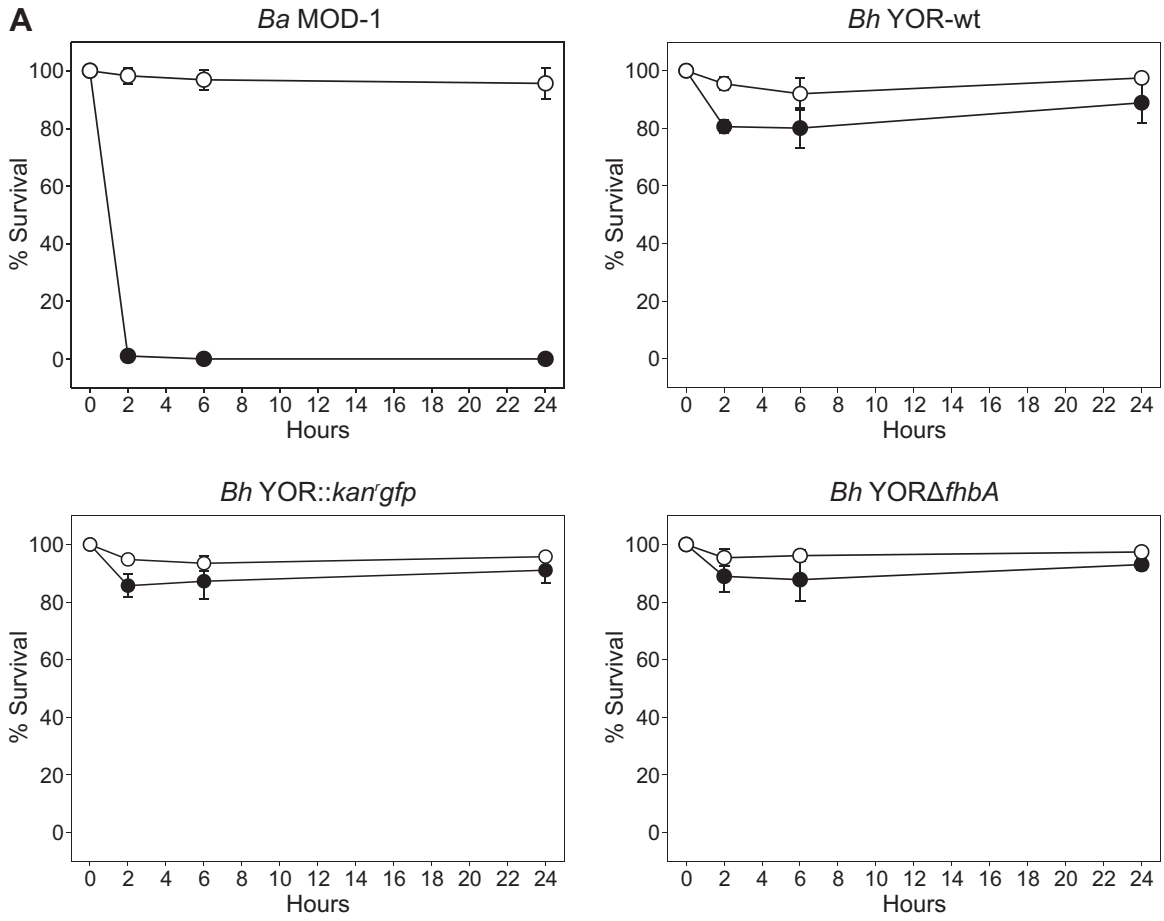
Although both *Bh* YORΔ*fhbA* and *B. andersonii* MOD-1 lack the ability to bind FH, the latter displayed a high degree of serum sensitivity while *Bh* YORΔ*fhbA* remained serum resistant. To explore this discrepancy, an assay was performed to assess cell-surface deposition of complement protein C9, an integral component of the cytolytic membrane attack complex (MAC), following incubation in serum. Cells were incubated in 10% NHS or hiNHS, washed, and screened by ELISA using anti-C9 antiserum. Consistent with its high degree of serum sensitivity, *B. andersonii* MOD-1 underwent significant C9 deposition in complement-active serum, while minimal deposition was seen on the FH-binding, serum resistant strains *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* (Figure 9B). Interestingly, *Bh* YORΔ*fhbA* did not undergo increased C9 deposition, suggesting a molecular basis for its ability to resist destruction in complement-active serum.

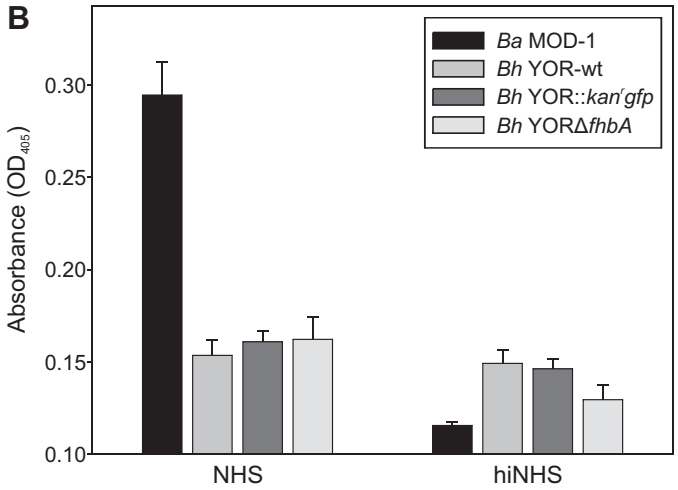
Demonstration of the ability of *B. hermsii* YOR transformants to infect and persist in mice

To determine if *B.hermsii* YOR transformant strains are infectious, mice (n=5) were needle inoculated and blood was collected at 0, 3, 4, 7, 10, 14, 18, and 42 days post-inoculation. Spirochetemia was measured by microscopic counts of organisms in 5 μl blood (diluted 1:20 in sodium citrate anticoagulant and PBS). All *B. hermsii* YOR strains were infectious and reached comparable levels in the bloodstream (Figure 10A). Only a single spirochetemic peak, on Day 4 of infection, was observed for all infecting strains. However, *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* persisted through Day 14, and *Bh* YORΔ*fhbA* through Day 18, in individual animals, as evidenced by re-cultivation of spirochetes from cultured blood samples (Table 3). All mice

Figure 9. Serum sensitivity analyses and assessment of C9 cell surface deposition. (A) Cells of *B. andersonii* MOD-1 (serum sensitive control) and each *B. hermsii* YOR strain were incubated with 50% normal human serum (NHS; closed circles) or heat-inactivated human serum (hiNHS; open circles). Cell viability was assessed after 2, 6, and 24 hours of incubation using *Bac*Light LIVE/DEAD staining and fluorescence microscopy. The mean percent survivals were calculated from counts of ten fields (400X magnification) per strain at each time point. (B) Deposition of complement component C9 on cells of *B. andersonii* MOD-1 (control) and *B. hermsii* strains was assessed by ELISA. Following incubation in 10% NHS or hiNHS, cells were immobilized into ELISA plates and screened with goat anti-C9 antiserum and an appropriate secondary antibody. Samples were run in triplicate, with error bars representing standard deviation.

Figure 9


A
Ba MOD-1
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA
% Survival
Hours
0
20
40
60
80
100
0 2 4 6 8 10 12 14 16 18 20 22 24


B
Ba MOD-1
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA
Absorbance (OD405)
0.10
0.15
0.20
0.25
0.30
NHS
hiNHS

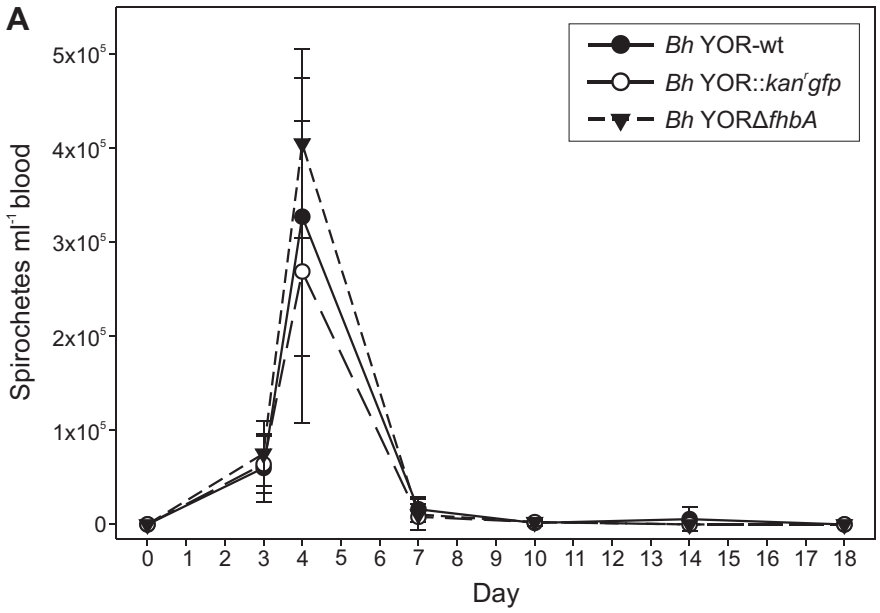
developed significant and equivalent anti-*B. hermsii* IgG titers, indicating seroconversion (Figure 10B). To verify the integrity of the infecting strains, an attempt was made to PCR amplify *fhbA* from each strain re-isolated from murine blood (obtained at peak spirochetemia, 4 days post-inoculation) using primers TC2 and TC3 (Table 2). Amplicons were generated from each *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* isolate, but not from any isolate of *Bh* YORΔ*fhbA* (Figure 11A). Additionally, screening of immunoblots of recombinant *B. hermsii* YOR FhbA (r-FhbA) with pooled infection sera from each experimental group demonstrated that an FhbA-specific IgG response was generated during infection with *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp*, but not during infection with *Bh* YORΔ*fhbA* (Figure 11B).

Demonstration of the *in vivo* stability of spirochetal GFP expression

To determine if the *Bh* YOR::*kan*^{r}*gfp* and *Bh* YORΔ*fhbA* transformant strains stably produce GFP during infection and upon re-isolation from mammals, fluorescence microscopy was performed on spirochetes recultivated from mouse blood in the absence of kanamycin (data not shown) and on diluted murine blood samples obtained during infection (Figure 12). All transformant spirochetes observed by microscopy produced GFP, demonstrating that GFP production by these strains is stable *in vivo* and upon release of antibiotic selection.

Figure 10. Analysis of infectivity and persistence in mice. Mice (n=5) were infected with *B. hermsii* YOR strains by needle inoculation. (A) At days 3, 4, 7, 10, 14, and 18 post-inoculation, peripheral blood samples were obtained and assessed for the presence of spirochetes by darkfield microscopy. Data are presented as number of spirochetes per ml of blood for each infecting strain at each time point. Calculations are detailed in the text. Error bars represent standard deviation. (B) To compare humoral immune responses elicited by each infecting strain (as indicated), murine anti-*B. hermsii* IgG titers were determined using serum harvested from mice 4 weeks post-inoculation and ELISA analysis. Whole cells of wild type *B. hermsii* YOR (*Bh* YOR-wt) served as the antigenic target. Experimental groups were compared based on geometric means (grey bars) of titers calculated for individual mice (triangles) at 1/3 maximum absorbance (OD_{405}).

Figure 10


A
Spirochetes ml-1 blood
5x10^5
4x10^5
3x10^5
2x10^5
1x10^5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Day
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA

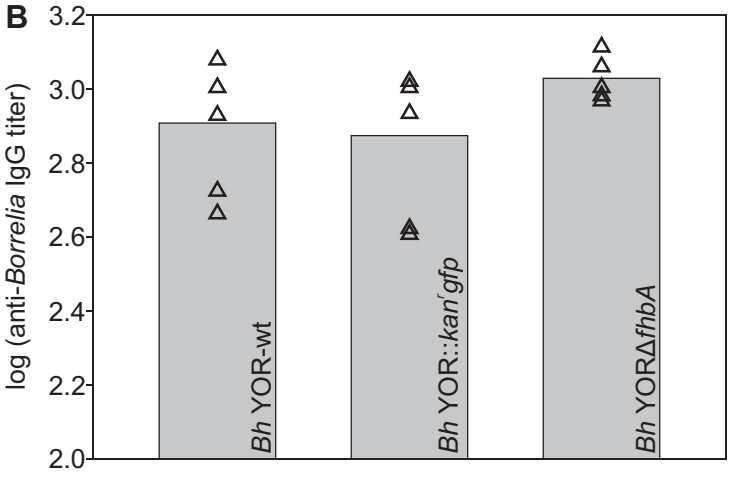

B
log (anti-Borrelia IgG titer)
3.2
3.0
2.8
2.6
2.4
2.2
2.0
Bh YOR-wt
Bh YOR::kanrgfp
Bh YORΔfhbA

Table 3. Recultivation of spirochetes from blood of infected mice

Strains	**0**	**3**	**4**	**7**	**10**	**14**	**18**	**42**
Bh YOR-wt	0/5	5/5	5/5	5/5	5/5	1/5	0/5	0/5
Bh YOR::*kan*^{r}*gfp*	0/5	5/5	5/5	5/5	4/5	2/5	0/5	0/5
Bh YORΔ*fhbA*	0/5	5/5	5/5	5/5	5/5	3/5	2/5	0/5

Data are shown as: (number of mice yielding positive blood cultures / total number of mice) at each time point (in **days**) post-inoculation

Figure 11

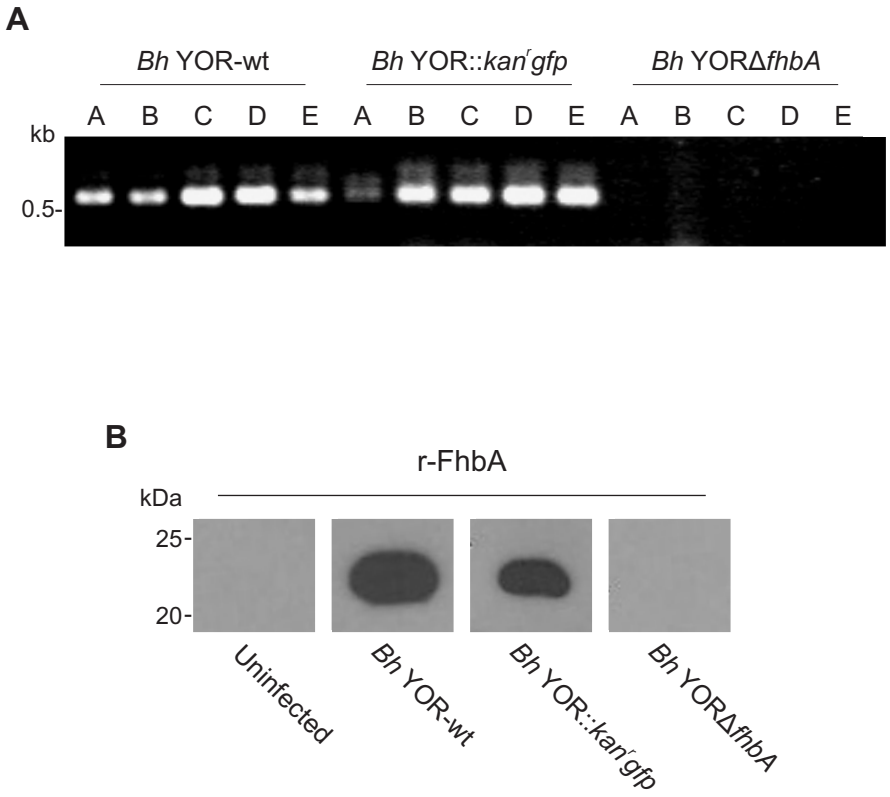


Figure 11. Serological and PCR-based analyses of infectious *B. hermsii* strains. (A) All *B. hermsii* YOR strains recultivated from the blood of individual mice (A-E, indicated above the panel) were analyzed by PCR using *fhbA*-specific primers (TC2/TC3; Table 2). Associated ethidium-bromide stained amplicons are shown. (B) The ability of each strain to elicit an FhbA-reactive IgG response during murine infection was assessed by screening identical r-FhbA immunoblots with infection sera (pooled by infecting strain, as indicated below each panel) obtained from mice 4 weeks post-inoculation.

Figure 12

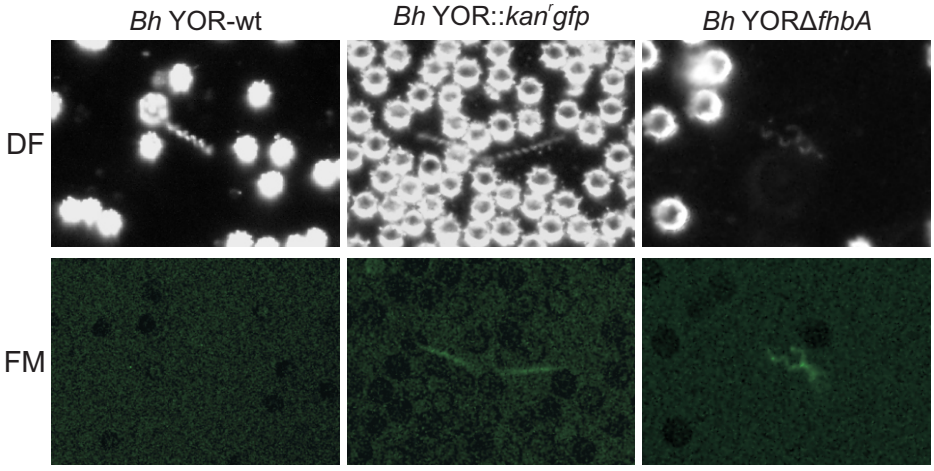


Figure 12. Demonstration of *in vivo* GFP production. Darkfield and fluorescence microscopy (DF and FM) were used to analyze spirochetal GFP production in diluted blood samples obtained from infected mice four days post-inoculation.

Discussion

Genetic manipulation techniques have been used to study multiple aspects of the biology and virulence of the LD spirochete *B. burgdorferi*. Prior to this study, however, there has been only a single report of successful genetic manipulation of a TBRF spirochete (17). Battisti and colleagues inactivated the *vtp* gene, which encodes the variable tick protein (Vtp), by replacing *vtp* with an antibiotic resistance cassette through site-directed allelic exchange. This study was performed using *B. hermsii* DAH clone 2E7 (a GGI strain) and standard methods of electro-transformation. While Vtp was found to be nonessential for infectivity in mammals, this earlier report served as the first demonstration that foreign DNA can be introduced in a site-specific manner into a TBRF spirochete.

In this work, we generated a clonal population of *B. hermsii* strain YOR that harbors a selectable marker (*kan*^{r}) and constitutively expresses a fluorescent marker (*gfp*). To achieve this, a suicide vector, pFAEV3, was constructed and electroporated into *B. hermsii* YOR. A major obstacle in the genetic manipulation of *B. burgdorferi* is that infectious strains often have very low transformation efficiencies (1-1000 transformants μg^{-1} DNA (91, 136), as opposed to $>10^7$ μg^{-1} DNA for *E. coli*) for reasons that remain unclear. The limitations imposed by this intractability can generally be overcome by use of very large amounts of transforming DNA (up to 50 μg per 10^9 cells) (166). Here, because the transformation efficiency of *B. hermsii* was unknown, plasmid DNA was utilized at a high concentration of 25 μg per reaction (~10^9 cells). Transformation of *B. hermsii* YOR resulted in the insertion of the *kan*^{r}*-gfp* cassette into a non-

coding sequence of lp200 between the *fhbA* gene and a hypothetical ORF. lp200 was chosen to harbor the integration site due, in part, to the availability of sequence for this plasmid and its wide distribution and stability among *B. hermsii* isolates (78, 79).

In our initial attempts to perform genetic transformation of *B. hermsii*, we employed the spectinomycin-streptomycin resistance gene *aadA*, fused to a *B. burgdorferi flgB* promoter (59), for selection. Although the utility of this cassette in *B. burgdorferi* has been well established (50, 59, 84), multiple (>20) individual attempts to generate *B. hermsii* YOR transformants using this resistance cassette were uniformly unsuccessful. Although *B. burgdorferi* B31 and *B. hermsii* DAH *flgB* promoters are very similar (94% sequence identity), the use of certain foreign promoters has been shown to decrease expression of antibiotic resistance genes in *Borrelia* (21). Therefore, we reconstructed our vector in order to incorporate a kanamycin resistance gene (*kan*^{r}) (used with success in *B. hermsii* previously (17)) linked to a native *B. hermsii* DAH *flgB* promoter. When the remodeled pFAEV3 vector was subsequently used in transformation experiments, 33% of electroporation reactions yielded positive transformants. It is not clear if the promoter element or the *kan*^{r} resistance gene (or both) enabled successful transformation, but no conditions beyond those were altered between experimental attempts.

Having successfully established a protocol for transformation of *B. hermsii* YOR, we reasoned that similar methods could be extended to study specific *B. hermsii* virulence mechanisms. Of particular interest was the immune evasive mechanism of FH binding. Many pathogens produce proteins able to bind FH at the cell surface, thereby exploiting its function as a negative regulator of the complement cascade (61, 74, 112, 113, 140). In several pathogenic spirochetes, including the *Borrelia*, FH-binding ability appears to correlate with complement

resistance and, potentially, infective potential (23, 76, 86, 101, 108, 131). However, direct evidence of the role of FH-binding in the Borrelial disease process is lacking.

B. hermsii is an ideal model system in which to study this process. For *B. burgdorferi*, which produces five factor H-binding proteins (CspA; CspZ; and OspE family proteins BBN38, BBL39, and BBP38), complete elimination of the FH-binding phenotype would require inactivation of multiple genes on various plasmids. While *cspA* (*bbA68*) and *cspZ* (*bbh06*) have been inactivated individually (23, 36), the inactivation of five Borrelial genes simultaneously is beyond the scope of current techniques. In contrast, *B. hermsii* produces only one factor H-binding protein, FhbA. *fhbA* is a single copy gene with no known paralogs, is transcribed as a monocistronic message, and is separated from putative upstream and downstream ORFs by >100 bases (78). Collectively, these factors recommended *fhbA* as a highly useful and feasible target for mutagenesis.

Genetic manipulation methods were repeated to generate a *B. hermsii* YOR *fhbA* deletion mutant (*Bh* YORΔ*fhbA*) using the pFAEV3Δ*fhbA* allelic exchange vector and electroporation. Transformation with this vector resulted in deletion of the *fhbA* coding region from lp200 and integration of the *kan*^{r}*-gfp* cassette through a double-crossover event.

Following transformation and recovery in liquid culture, clonal populations of *Bh* YOR::*kan*^{r}*gfp* and *Bh* YORΔ*fhbA* were derived through limiting dilution. For unknown reasons, efforts to generate clonal populations using the standard sub-surfacing plating methods developed for *B. burgdorferi* (136) were not successful. PCR analyses and fluorescence microscopy were performed to verify successful transformation and GFP expression.

An additional challenge in the genetic manipulation of *B. burgdorferi* is loss of endogenous plasmids that encode proteins required for survival in either tick or mammalian

hosts (91, 124, 132). In *B. burgdorferi*, plasmid content is typically assessed by PCR using plasmid-specific primer sets (87, 105, 124). However, while the sequence of the *B. hermsii* DAH chromosome is available, sequences for the plasmids are not. Hence, plasmid-specific PCR could not be applied for this purpose here. Instead, plasmid profiles of the *B. hermsii* YOR wild type and transformant strains were assessed by pulsed field gel electrophoresis (PFGE). We did not observe any differences in plasmid profiles between strains, although it is possible that some *B. hermsii* plasmids were not discerned by this approach due to size similarities and co-migration patterns. Additionally, growth curve analyses demonstrated comparable growth rates between transformant and parental strains. This verified that neither insertion and expression of *kan^{r}gfp* nor deletion of *fhbA* interfere with *in vitro* viability, and provided additional evidence that plasmids necessary for growth were not lost during transformation.

To analyze expression and cell-surface presentation of FhbA, cells of each strain were incubated with or without Proteinase K, then fractionated by SDS-PAGE. Immunoblot analyses using antiserum directed against FhbA demonstrated that while *Bh* YOR::*kan^{r}gfp* expressed FhbA at the cell surface, FhbA was not expressed by *Bh* YORΔ*fhbA*. To further assess FhbA/factor H-binding protein expression, untreated cell lysates were fractionated by SDS-PAGE and analyzed by FH ALBI. This assay detects membrane-immobilized spirochetal proteins capable of binding FH. As anticipated, *Bh* YOR-wt and *Bh* YOR::*kan^{r}gfp* bound FH to a single ~20 kDa protein consistent in size with FhbA, while no proteins produced by the *fhbA* deletion mutant displayed FH-binding ability.

One limitation of the FH ALBI assay as performed here is that reducing and denaturing conditions may alter sample properties and conformation prior to analysis. Therefore, it is possible that certain proteins capable of binding FH under nondenaturing, physiologic conditions

may not be detected by this method. The *Bh* YORΔ*fhbA* mutant strain provides a unique opportunity to examine physiologic *B. hermsii* FH binding through a whole cell adsorption assay. Here, cells of each strain were incubated in purified human FH, washed, then screened for cell-surface FH adsorption using anti-FH antibodies. Both IFA and immunoblot screening methods demonstrated that FhbA-positive, but not FhbA-negative, cells adsorbed FH to the cell surface. This result provides the first direct evidence that FhbA is *B. hermsii's* sole factor H binding protein and that its deletion results in complete loss of the FH-binding phenotype. Consistent with this, *Bh* YORΔ*fhbA* was unable to cleave and inactivate complement protein C3b in the presence of factor I and following incubation with factor H. This almost certainly is a functional consequence of its inability to bind FH. Data here and in previous studies demonstrate that *B. hermsii* YOR lacks inherent C3b cleavage abilities (e.g., through production of proteases), but rather binds FH in a functionally active manner that permits cofactor activity in the factor-I mediated cleavage of C3b (106).

In addition to FH, FhbA has been shown to interact with multiple host ligands including plasminogen (76). To assess the contribution of FhbA to whole-cell plasminogen binding, plate-immobilized cells of each strain were incubated with plasminogen, washed, and screened using anti-plasminogen antibodies. This binding assay revealed that levels of retained plasminogen appeared marginally decreased for *Bh* YORΔ*fhbA* as compared to *Bh* YOR-wt; however, all strains demonstrated clear plasminogen-binding ability as compared to controls. This suggests that although FhbA may contribute to cumulative plasminogen acquisition by *B. hermsii* YOR, alternate mechanisms likely compensate for the loss of FhbA expression. Therefore, an attempt to identify additional *B. hermsii* plasminogen-binding proteins may be warranted.

Previous studies have demonstrated that *B. hermsii* high passage strain REN, which lacks *fhbA*, exhibits increased sensitivity to killing in serum and an inability to persist in, but not to infect, mice (76). To examine whether deletion of *fhbA* in strain YOR similarly impacts serum resistance, all *B. hermsii* YOR strains, as well as *B. andersonii* MOD-1 (a non-FH-binding, serum sensitive control strain) were incubated in normal, complement-active human serum (NHS) or in serum rendered complement-inactive through heat treatment (hiNHS). Intriguingly, while *B. andersonii* was rapidly killed in complement-active NHS, all *B. hermsii* YOR strains exhibited a high degree of serum resistance in NHS out to 24 hours. Analyses of cell-surface C9 deposition on similarly treated cells provided insight into the molecular basis of these observations, as *B. andersonii* MOD-1 was found to bear high levels of membrane-associated C9, a terminal MAC component. All *B. hermsii* strains, however, including *Bh* YORΔ*fhbA*, were spared such deposition. Consistent with these results, infection analyses in C3H/HeJ mice demonstrated *Bh* YORΔ*fhbA* to be fully infectious, generating spirochetemias and eliciting anti-*B. hermsii* IgG responses equivalent to *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp*. Unlike FhbA-negative *B. hermsii* strain REN, which reached high levels during initial infection then was prematurely cleared, *Bh* YORΔ*fhbA* was cultured from the blood of individual mice at Day 18, a time point beyond when *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* had been eliminated from their respective hosts. Although only moderately reliable in the clinical setting (5, 62, 116, 137, 154), direct microscopic examinations of peripheral blood samples are widely used to monitor RF infections under laboratory conditions (4, 65, 95, 152) where disease kinetics can be anticipated and assessed over a broad temporal range. Nonetheless, sample size inherently constrains the sensitivity of this method (46), and the detection limit here was approximately 4×10^3 spirochetes ml^{-1} murine blood. Direct blood culturing, when successful, offers increased

detection sensitivity (here, a theoretical limit of 167 spirochetes ml^{-1} blood), but not quantitative assessment of spirochete burden. Coupling of these assays allowed us to assess and compare various aspects of infections produced by each *B. hermsii* strain. Although highly sensitive PCR-based molecular diagnostic techniques for RF have been developed (typically targeting the 16S rRNA or *glpQ* genes, or the *rrs–rrlA* intergenic spacer region (5, 116, 137)), they are cost- and labor-intensive and thus are not typically employed for routine spirochetemic monitoring.

Because limiting dilution is not a standard method of establishing clonal populations in the *Borrelia*, additional assays were performed to ensure that the observed infections were in fact caused by transformant populations and not by the rare wild type variant that was able to proliferate in the *in vivo* environment. PCR analysis demonstrated that *fhbA* could be amplified from all strains recultivated from *Bh* YOR-wt- and *Bh* YOR::*kan*^{r}*gfp*- infected mice, but not from strains recultivated from mice infected with *Bh* YORΔ*fhbA*. Consistent with this, sera from mice infected with *Bh* YOR-wt and *Bh* YOR::*kan*^{r}*gfp* were reactive against recombinant FhbA protein, but sera from YORΔ*fhbA*-infected mice and from uninfected mice were nonreactive. Stable expression of GFP was observed for all transformant strains both in murine blood samples during infection and in culture (without kanamycin selection) following recultivation from blood. This provides important confirmation that the *gfp* insertion is stably maintained in the *B. hermsii* genome.

In summary, *Bh* YORΔ*fhbA* does not produce FhbA or bind factor H. Because it lacks FH-binding abilities, this strain is unable to cleave and inactivate C3b in solution. However, *Bh* YORΔ*fhbA* is not susceptible to C9 deposition, as is reflected in its high degree of serum resistance, and is infectious and able to persist in a murine host. This suggests that *B. hermsii* can evade complement activation via one or multiple non-FH-mediated mechanisms. Such potential

mechanisms may involve binding or production of additional factors capable of negatively regulating the complement cascade. Although pathogen exploitation of FH in particular has been heavily scrutinized, studies have identified a plethora of additional evasive mechanisms involving host complement regulatory proteins (89). These proteins include both early (complement receptor type 1 (CR1), membrane cofactor protein (MCP), decay-accelerating factor (DAF)) and late (vitronectin, CD-59) regulators of the alternative complement pathway (Figure 13). Membrane-associated regulators, such as CR1, MCP, DAF, and CD-59, are broadly distributed across the surfaces of most mammalian host cells, where they act to control local complement activation along with performing a variety of additional functions. Important fluid-phase regulators of alternative complement activation include both FH and vitronectin, soluble proteins present in host plasma and some body fluids (175). Actions of alternative pathway regulatory proteins fall into three general groups: inactivation of C3b through factor I-mediated cleavage, destabilization of the C3bBb C3 convertase, and interference with terminal component assembly/membrane insertion (110) (Figure 13). Based on the results of the C3b assay presented here (Figure 7), *B. hermsii* YOR does not appear to produce endogenous factors that fall into the first category (i.e., participate in factor I-mediated C3b cleavage). Of particular interest, however, are the potential roles in *B. hermsii* complement evasion of vitronectin or a CD-59-like protein, both of which interact with terminal complement components to prevent MAC formation (110). The former would represent a novel mechanism in *Borrelia* (although it is employed by other pathogens, such as *Moraxella catarrhalis* (6, 153)), while the latter has been documented previously in *B. burgdorferi* (121).

In conclusion, these results collectively suggest that TBRF spirochete *B. hermsii* YOR employs an unidentified, FH-independent mechanism of complement evasion that is sufficient

Figure 13

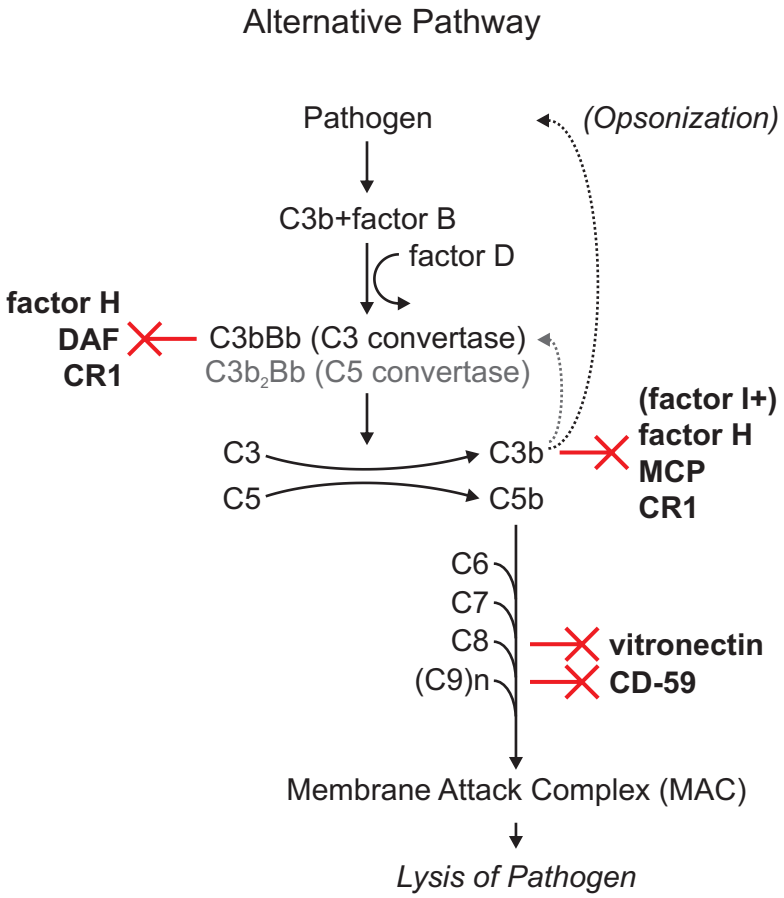


Figure 13. Negative regulators of the alternative complement pathway. Fluid-phase regulator factor H and cell-surface regulators CR1, DAF, and MCP inhibit alternative complement activation through both cofactor activity in the factor I-mediated cleavage of C3b and decay acceleration/destabilization of the C3 convertase (C3bBb). Vitronectin, a soluble regulator, prevents MAC formation by interacting with and blocking membrane insertion of the terminal C3b5-8 assemblage. Membrane protein CD-59 inhibits MAC formation by interacting with membrane-associated C3b5-8/-9 and preventing C9 incorporation and polymerization. These mechanisms are employed by the host to prevent inappropriate and injurious complement activation, but can be exploited by pathogens during the infectious process.

for persistence within the host. Whether this mechanism is direct and non-redundant, or multifactorial and complex, continued analyses of *Bh* YORΔ*fhbA* and generation of novel RF spirochete mutants may prove critical to its elucidation.

Literature Cited

Literature Cited

1. **Alitalo, A., T. Meri, H. Lankinen, I. Seppala, P. Lahdenne, P. S. Hefty, D. Akins, and S. Meri.** 2002. Complement inhibitor factor H binding to Lyme disease spirochetes is mediated by inducible expression of multiple plasmid-encoded outer surface protein E paralogs. J Immunol **169:**3847-3853.

2. **Alugupalli, K. R., et al.** 2001. Platelet activation by a relapsing fever spirochaete results in enhanced bacterium-platelet interaction via integrin $alpha_{IIb}beta_3$ activation. Mol Microbiol **39:**330-340.

3. **Alugupalli, K. R., R. M. Gerstein, J. Chen, E. Szomolanyi-Tsuda, R. T. Woodland, and J. M. Leong.** 2003. The resolution of relapsing fever Borreliosis requires IgM and is concurrent with expansion of B1b lymphocytes. J Immunol **170:**3819-3827.

4. **Alugupalli, K. R., A. D. Michelson, I. Joris, T. G. Schwan, K. Hodivala-Dilke, R. O. Hynes, and J. M. Leong.** 2003. Spirochete-platelet attachment and thrombocytopenia in murine relapsing fever borreliosis. Blood **102:**2843-2850.

5. **Assous, M. V., and A. Wilamowski.** 2009. Relapsing fever borreliosis in Eurasia---forgotten, but certainly not gone! Clin Microbiol Infect **1:**407-414.

6. **Attia, A. S., S. Ram, P. A. Rice, and E. J. Hansen.** 2006. Binding of vitronectin by the *Moraxella catarrhalis* UspA2 protein interferes with late stages of the complement cascade. Infect Immun **74:**1597-1611.

7. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J.-C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int J Syst Bacteriol **42:**378-383.

8. **Barbosa, A. S., P. A. Abreu, S. A. Vasconcellos, Z. M. Morais, A. P. Goncales, A. S. Silva, M. R. Daha, and L. Isaac.** 2009. Immune evasion of *Leptospira* species by acquisition of human complement regulator C4BP. Infect Immun **77:**1137-1143.

9. **Barbour, A. G.** 1990. Antigenic variation of a relapsing fever *Borrelia* species. Annu Rev Microbiol **44:**155-171.

10. **Barbour, A. G.** 1988. Plasmid analysis of *Borrelia burgdorferi*, the Lyme disease agent. J Clin Microbiol **26:**475-478.

11. **Barbour, A. G., and V. Bundoc.** 2001. In vitro and in vivo neutralization of the relapsing fever agent *Borrelia hermsii* with serotype-specific immunoglobulin M antibodies. Infect Immun **69:**1009-1015.

12. **Barbour, A. G., Q. Dai, B. I. Restrepo, H. G. Stoenner, and S. A. Frank.** 2006. Pathogen escape from host immunity by a genome program for antigenic variation. P Natl Acad Sci USA **103:**18290-18295.

13. **Barbour, A. G., and C. F. Garon.** 1987. Linear plasmids of the bacterium *Borrelia burgdorferi* have covalently closed ends. Science **237:**409-411.

14. **Barbour, A. G., and S. F. Hayes.** 1986. Biology of *Borrelia* species. Microbiol Rev **50:**381-400.

15. **Barbour, A. G., S. L. Tessier, and H. G. Stoenner.** 1982. Variable major proteins of *Borrellia hermsii*. J Exp Med **156:**1312-1324.

16. **Baril, C., C. Richaud, G. Baranton, and I. Saint Girons.** 1989. Linear chromosome of *Borrelia burgdorferi*. Res Microbiol **140:**507-516.

17. **Battisti, J. M., S. J. Raffel, and T. G. Schwan.** 2008. A system for site-specific genetic manipulation of the relapsing fever spirochete *Borrelia hermsii*. Methods Mol Biol **431:**69-84.

18. **Berggard, K., E. Johnsson, F. R. Mooi, and G. Lindahl.** 1997. *Bordetella pertussis* binds the human complement regulator C4BP: role of filamentous hemagglutinin. Infect Immun **65:**3638-3643.

19. **Blom, A. M., A. Rytkonen, P. Vasquez, G. Lindahl, B. Dahlback, and A.-B. Jonsson.** 2001. A novel interaction between type IV pili of *Neisseria gonorrhoeae* and the human complement regulator C4b-binding protein. J Immunol **166:**6764-6770.

20. **Bolz, D. D., R. S. Sundsbak, Y. Ma, S. Akira, J. H. Weis, T. G. Schwan, and J. J. Weis.** 2006. Dual role of MyD88 in rapid clearance of relapsing fever *Borrelia* spp. Infect Immun **74:**6750-6760.

21. **Bono, J. L., A. F. Elias, J. J. Kupko III, B. Stevenson, K. Tilly, and P. Rosa.** 2000. Efficient targeted mutagenesis in *Borrelia burgdorferi*. J Bacteriol **182:**2445-2452.

22. **Boyer, K. M., R. S. Munford, G. O. Maupin, C. P. Pattison, M. D. Fox, A. M. Barnes, W. L. Jones, and J. E. Maynard.** 1977. Tick-borne relapsing fever: an interstate outbreak originating at Grand Canyon National Park. Am J Epidemiol **105:**469-479.

23. **Brooks, C. S., S. R. Vuppala, A. M. Jett, A. Alitalo, S. Meri, and D. R. Akins.** 2005. Complement Regulator-Acquiring Surface Protein 1 Imparts Resistance to Human Serum in *Borrelia burgdorferi*. J Immunol **175:**3299-3308.

24. **Bryceson, A., E. Parry, P. Perine, D. Warrel, D. Vukotich, and C. Leithead.** 1970. Louse-borne relapsing fever: A clinical and laboratory study of 62 cases in Ethiopia and a reconsideration of the literature. Q J Med **39:**129-170.

25. **Burgdorfer, W., and A. J. Mavros.** 1970. Susceptibility of various species of rodents to the relapsing fever spirochete, *Borrelia hermsii*. Infect Immun **2:**256-259.

26. **Burman, N., A. Shamaei-Tousi, and S. Bergstrom.** 1998. The spirochete *Borrelia corcidurae* causes erythrocyte rosetting during relapsing fever. Infect Immun **66:**815-819.

27. **Cadavid, D., and A. G. Barbour.** 1998. Neuroborreliosis during relapsing fever: Review of the clinical manifestations, pathology and treatment of infections in humans and experimental animals. Clin Infect Dis **26:**151-164.

28. **Cadavid, D., A. R. Pachner, L. Estanislao, R. Patalapati, and A. G. Barbour.** 2001. Isogenic serotypes of *Borrelia turicatae* show different localization in the brain and skin of mice. Infect Immun **69:**3389-3397.

29. **Carlyon, J. A., C. LaVoie, S. Y. Sung, and R. T. Marconi.** 1998. Analysis of the organization of multicopy linear- and circular-plasmid-carried open reading frames in *Borrelia burgdorferi* sensu lato isolates. Infect Immun **66:**1149-1158.

30. **Casjens, S., M. DeLang, H. L. Ley, III, P. A. Rosa, and W. M. Huang.** 1995. Linear chromosome of Lyme disease agent spirochetes: Genetic diversity and conservation of gene order. J Bacteriol **177:**2769-2780.

31. **Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser.** 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. Mol Microbiol **35:**490-516.

32. **CDC.** 2010. Summary of notifiable diseases-United States, 2008. 2010. MMWR **54**:76-85.

33. **Chaconas, G., P. E. Stewart, K. Tilly, B. J. L., and P. Rosa.** 2001. Telomere resolution in the Lyme disease spirochete. J Embo **20:**3229-3237.

34. **Charon, N. W., S. F. Goldstein, M. Marko, C. Hsieh, L. L. Gebhardt, M. A. Motaleb, C. W. Wolgemuth, R. J. Limberger, and N. Rowe.** 2009. The flat-ribbon

configuration of the periplasmic flagella of *Borrelia burgdorferi* and its relationship to motility and morphology. J Bacteriol **191:**600-607.

35. **Coburn, J., J. M. Leong, and J. K. Erban.** 1993. Integrin alpha IIb beta 3 mediates binding of the Lyme disease agent *Borrelia burgdorferi* to human platelets. P Natl Acad Sci USA **90:**7059-7063.

36. **Coleman, A. S., X. Yang, M. Kumar, X. Zhang, K. Promnares, D. Shroder, M. R. Kenedy, J. F. Anderson, D. R. Akins, and U. Pal.** 2008. *Borrelia burgdorferi* complement regulator-acquiring surface protein 2 does not contribute to complement resistance or host infectivity. PLoS ONE **3:**3010e.

37. **Coleman, J. L., J. A. Gebbia, J. Piesman, J. L. Degen, T. H. Bugge, and J. L. Benach.** 1997. Plasminogen is required for efficient dissemination of *Borrelia burgdorferi* in ticks and for enhancement of spirochetemia in mice. Cell **89:**1111-1119.

38. **Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach.** 1995. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. Infect Immun **63:**2478-2484.

39. **Colombo, M. J., and K. R. Alugupalli.** 2008. Complement factor H-binding protein, a putative virulence determinant of *Borrelia hermsii*, is an antigenic target for protective B1b lymphocytes. J Immunol **180:**4858-4864.

40. **Connolly, S. E., D. G. Thanassi, and J. L. Benach.** 2004. Generation of a Complement-Independent Bactericidal IgM against a Relapsing Fever *Borrelia*. J Immunol **172:**1191-1197.

41. **Cutler, S., and A. Talbert.** 2003. Tick-borne relapsing fever in Tanzania -- a forgotten problem? ASM News **69:**542-543.

42. **Cutler, S. J.** 2006. Possibilities for replapsing fever reemergence. Emerg Infect Dis **12:**369-374.

43. **Dai, Q., B. I. Restrepo, S. F. Porcella, S. J. Raffel, T. G. Schwan, and A. G. Barbour.** 2006. Antigenic variation by *Borrelia hermsii* occurs through recombination between extragenic repetitive elements on linear plasmids. Mol Microbiol **60:**1329-1343.

44. **Dennis, D. T.** 2008. Relapsing Fever. *In* A. S. Fauci, MD (ed.), Harrison's Principles of Internal Medicine, 17 ed. The McGraw-Hill Companies, Inc., Printed in the United States of America.

45. **Dupont, H. T., B. La Scola, R. Williams, and D. Raoult.** 1997. A focus of tick-borne relapsing fever in southern Zaire. Clin Infect Dis **25:**139-144.

46. **Dworkin, M. S.** 2002. Tick-borne relapsing fever in North America. Med Clin North Am **86:**417-433.

47. **Dworkin, M. S., J. Anderson, D. E., T. G. Schwan, P. C. Shoemaker, S. N. Banerjee, B. O. Kassen, and W. Burgdorfer.** 1998. Tick-borne relapsing fever in the Northwestern United States and Southwestern Canada. Clin Infect Dis **26:**122-131.

48. **Dworkin, M. S., T. G. Schwan, D. E. Anderson, and S. M. Borchardt.** 2008. Tick-bourne relapsing fever. Infect Dis Clin N Am **22:**449-468.

49. **Dworkin, M. S., P. C. Shoemaker, C. L. Fritz, M. E. Dowell, and D. E. Anderson, Jr.** 2002. The epidemiology of tick-borne relapsing fever in the United States. Am J Trop Med Hyg **66:**753-758.

50. **Earnhart, C. G., D. V. Leblanc, K. E. Alix, D. C. Desrosiers, J. D. Radolf, and R. T. Marconi.** 2010. Identification of residues within ligand-binding domain 1 (LBD1) of the *Borrelia burgdorferi* OspC protein required for function in the mammalian environment. Mol Microbiol **76:**393-408.

51. **Eggers, C. H., M. J. Caimano, and J. D. Radolf.** 2004. Analysis of promoter elements involved in the transcriptional initiation of RpoS-dependent *Borrelia burgdorferi* genes. J Bacteriol **186:**7390-7402.

52. **Evans, N. J., K. Brown, D. Timofte, V. R. Simpson, and R. J. Birtles.** 2009. Fatal Borreliosis in bat caused by relapsing fever spirochete, United Kingdom. Emerg Infect Dis **15:**1331-1332.

53. **Fekade, D., K. Knox, K. Hussein, A. Melka, D. G. Lalloo, R. Coxon, and D. A. Warrell.** 1996. Prevention of Jarisch-Herxheimer reactions by treatment with antibodies against tumor necrosis factor α. New Engl J Med **335:**311-315.

54. **Felsenfeld, O.** 1971. *Borrelia*. Strains, vectors, human and animal borreliosis. Warren H. Green, Inc., St. Louis.

55. **Felsenfeld, O.** 1965. *Borreliae*, human relapsing fever, and parasite-vector-host relationships. Bacteriol Rev **29:**46-73.

56. **Ferdows, M. S., and A. G. Barbour.** 1989. Megabase-sized linear DNA in the bacterium *Borrelia burgdorferi*, the Lyme disease agent. P Natl Acad Sci USA **86:**5969-5973.

57. **Ferreira, V. P., A. P. Herbert, H. G. Hocking, P. N. Barlow, and M. K. Pangburn.** 2006. Critical role of the C-terminal domains of factor H in regulating complement activation at cell surfaces. J Immunol **177:**6308-6316.

58. **Fischer, R. J., T. L. Johnson, S. J. Raffel, and T. G. Schwan.** 2009. Identical strains of *Borrelia hermsii* in mammal and bird. Emerg Infect Dis **15:**2064-2066.

59. **Frank, K. L., S. F. Bundle, M. E. Kresge, C. H. Eggers, and D. S. Samuels.** 2003. *aadA* confers streptomycin resistance in *Borrelia burgdorferi*. J Bacteriol **185:**6723-6727.

60. **Fraser, C. M., et al.** 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. Nature **390:**580-586.

61. **Friberg, N., P. Carlson, E. Kentala, P. S. Mattila, P. Kuusela, S. Meri, and H. Jarva.** 2008. Factor H binding as a complement evasion mechanism for an anaerobic pathogen, *Fusobacterium necrophorum*. J Immunol **181:**8624-8632.

62. **Fritz, C. L., L. R. Bronson, C. R. Smith, M. E. Schriefer, J. R. Tucker, and T. G. Schwan.** 2004. Isolation and characterization of *Borrelia hermsii* associated with two foci of tick-borne relapsing fever in California. J Clin Microbiol **42:**1123-1128.

63. **Fuchs, H., R. Wallich, M. M. Simon, M. D. Kramer.** 1994. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. P Natl Acad Sci USA **91:**12594-12598.

64. **Galbe, J. L., E. Guy, J. M. Zapatero, E. I. B. Peerschke, and J. L. Benach.** 2002. Vascular clearance of *Borrelia burgdorferi* in rats. Microb Pathogenesis **14:**187-201.

65. **Gebbia, J. A., J. C. G. Monco, J. L. Degen, T. H. Bugge, and J. L. Benach.** 1999. The plasminogen activation system enhances brain and heart invasion in murine relapsing fever borreliosis. J Clin Invest **103:**81-87.

66. **Goldstein, S. F., N. W. Charon, and J. A. Kreiling.** 1994. *Borrelia burgdorferi* swims with a planar waveform similar to that of eukaryotic flagella. P Natl Acad Sci USA **91:**3433-3437.

67. **Goubau, P. F.** 1984. Relapsing fevers. A review. Ann Soc Belg Med Trop **64:**335-364.

68. **Grab, D.** 2005. *Borrelia burgdorferi*, host-derived proteases and the blood brain barrier. Infect Immun **73:**1014-1022.

69. **Grosskinsky, S., M. Schott, C. Brenner, S. J. Cutler, P. Kraiczy, P. F. Zipfel, M. M. Simon, and R. Wallich.** 2009. *Borrelia recurrentis* employs a novel multifunctional surface protein with anti-complement, anti-opsonic and invasive potential to escape innate immunity. PLoS ONE **4:**e4858.

70. **Guo, B. P., S. Teneberg, R. Munch, D. Terunuma, K. Hatano, K. Matsuoka, J. Angstrom, T. Boren, and S. Bergstrom.** 2209. Relapsing fever *Borrelia* binds to

neolacto glycans and mediates rosetting of human erythrocytes. P Natl Acad Sci **106:**19280-19285.

71. **Hanincova, K., K. Kurtenbach, M. Diuk-Wasser, B. Brei, and D. Fish.** 2006. Epidemic spread of Lyme borreliosis, northeastern United States. Emerg Infect Dis **12:**604-611.

72. **Harboe, M. H., and T. E. Mollnes.** 2008. The alternative complement pathway revisited. J Cell Mol Med **12:**1074-1084.

73. **Hartmann, K., C. Corvey, C. Skerka, M. Kirschfink, M. Karas, V. Brade, J. C. Miller, B. Stevenson, R. Wallich, P. F. Zipfel, and P. Kraiczy.** 2006. Functional characterization of BbCRASP-2, a distinct outer membrane protein of *Borrelia burgdorferi* that binds host complement regulators factor H and FHL-1. Mol Microbiol **61:**1220-1236.

74. **Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti.** 1988. Antiphagocytic acitivity of streptoccocal M protein: selective binding of complement control protein factor H. P Natl Acad Sci USA **85:**1657-1661.

75. **Houhamdi, L., and D. Raoult.** 2005. Excretion of living *Borrelia recurrentis* in feces of infected human body lice. J Infect Dis **191:**1898-1906.

76. **Hovis, K. M., J. C. Freedman, H. Zhang, J. L. Forbes, and R. T. Marconi.** 2008. Identification of an antiparallel coiled-coil/loop domain required for ligand binding by the *Borrelia hermsii* FhbA protein: additional evidence for the role of FhbA in the host-pathogen interaction. Infect Immun **76:**2113-2122.

77. **Hovis, K. M., J. P. Jones, T. Sadlon, G. Raval, D. L. Gordon, and R. T. Marconi.** 2006. Molecular analyses of the interaction of *Borrelia hermsii* FhbA with the complement regulatory proteins factor H and factor H-like protein 1. Infect Immun **74:**2007-2014.

78. **Hovis, K. M., J. V. McDowell, L. Griffin, and R. T. Marconi.** 2004. Identification and characterization of a linear-plasmid-encoded factor H-binding protein (FhbA) of the relapsing fever spirochete *Borrelia hermsii*. J Bacteriol **186:**2612-2618.

79. **Hovis, K. M., M. E. Schriefer, S. Bahlani, and R. T. Marconi.** 2006. Immunological and molecular analyses of the *Borrelia hermsii* factor H and factor H-like protein 1 binding protein, FhbA: Demonstration of its utility as a diagnostic marker and epidemiological tool for tick-borne relapsing fever. Infect Immun **74:**4519-4529.

80. **Hovis, K. M., E. Tran, C. M. Sundy, E. Buckles, J. V. McDowell, and R. T. Marconi.** 2006. Selective binding of *Borrelia burgdorferi* OspE paralogs to factor H and serum proteins from diverse animals: possible expansion of the role of OspE in Lyme disease pathogenesis. Infect Immun **74:**1967-1972.

81. **Hu, L., S. D. Pratt, G. Perides, L. Katz, R. A. Rogers, and M. S. Klempner.** 1997. Isolation, cloning, and expression of a 70-kilodalton plasminogen binding protein of *Borrelia burgdorferi*. Infect Immun **65:**4989-4995.

82. **Jongen, V. H., J. van Roosmalen, J. Tiems, J. Van Holten, and J. C. Wetsteyn.** 1997. Tick-borne relapsing fever and pregnancy outcome in rural Tanzania. Acta Obstet Gynecol Scand **76:**834-838.

83. **Kitten, Y., and A. G. Barbour.** 1992. The relapsing fever agent *Borrelia hermsii* has multiple copies of its chromosome and linear plasmids. Genetics **132:**311-324.

84. **Kostick, J. L., L. T. Szokotnicki, E. A. Rogers, P. Bocci, N. Raffaelli, and R. T. Marconi.** 2011. The diguanylate cyclase, Rrp1, regulates critical steps in the enzootic cycle of the Lyme disease spirochetes. Mol Microbiol **Accepted Article:**doi: 10.1111/j.1365-2958.2011.07687.x.

85. **Kudryashev, M., M. Cyrklaff, W. Baumeister, M. M. Simon, R. Wallich, and F. Frischknecht.** 2009. Comparative cryo-electron tomography of pathogenic Lyme disease spirochetes. Mol Microbiol **71:**1415-1434.

86. **Kurtenbach, K., H. S. Sewell, N. H. Ogden, S. E. Randolph, and P. A. Nuttall.** 1998. Serum complement sensitivity as a key factor in Lyme disease ecology. Infect Immun **66:**1248-1251.

87. **Labandeira-Rey, M., and J. T. Skare.** 2001. Decreased infectivity in *Borrelia burgorferi* strain B31 is associated with the loss of either linear plasmid 25 or 28-1. Infect Immun **69:**446-455.

88. **Lagal, V., D. Portnoi, G. Faure, D. Postic, and G. Baranton.** 2006. *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. Microbes Infect **8:**645-652.

89. **Lambris, J. D., D. Ricklin, and B. V. Geisbrecht.** 2008. Complement evasion by human pathogens. Nat Rev Microbiol **6**.

90. **Larsson, C., M. Andersson, J. Pelkonen, B. P. Guo, A. Nordstrand, and S. Bergstrom.** 2006. Persistent brain infection and disease reactivation in relapsing fever borreliosis. Microbes Infect **8:**2213-2219.

91. **Lawrenz, M. B., H. Kawabata, J. E. Purser, and S. J. Norris.** 2002. Decreased electroporation efficiency in *Borrelia burgdorferi* containing lineaer plasmids lp25 and lp56: Impact on transformation of infectious *B. burgdorferi*. Infect Immun **70:**4798-4804.

92. **Lescot, M., S. Audic, C. Robert, T. T. Nguyen, G. Blanc, S. J. Cutler, P. Wincker, A. Couloux, J. M. Claverie, D. Raoult, and M. Drancourt.** 2008. The genome of *Borrelia*

recurrentis, the agent of deadly louse-borne relapsing fever, is a degraded subset of tick-borne *Borrelia duttonii*. PLoS Genet **4:**e1000185.

93. **Lin, T., J. H. Oliver, Jr., L. Gao, T. M. Kollars, Jr., and K. L. Clark.** 2001. Genetic heterogeneity of *Borrelia burgdorferi* sensu lato in the southern United States based on restriction fragment length polymorphism and sequence analysis. J Clin Microbiol **39:**2500-2507.

94. **Londono, D., Y. Bai, W. R. Zuckert, H. Gelderblom, and D. Cadavid.** 2005. Cardiac apoptosis in severe relapsing fever borreliosis. Infect Immun **73:**7669-7676.

95. **Lopez, J. E., M. E. Schrumpf, S. J. Raffel, P. F. Policastro, S. F. Porcella, and T. G. Schwan.** 2008. Relapsing fever spirochetes retain infectivity after prolonged in vitro cultivation. Vector Borne Zoonotic Dis **8:**813-820.

96. **Lundqvist, J., C. Larsson, M. Nelson, M. Andersson, S. Bergstrom, and C. Persson.** 2010. Concomitant infection decreases the malaria burden but escalates relapsing fever Borreliosis. Infect Immun **78:**1924-1930.

97. **Marconi, R. T., and C. F. Garon.** 1992. Phylogenetic analysis of the genus *Borrelia*: a comparison of North American and European isolates of *Borrelia burgdorferi*. J Bacteriol **174:**241-244.

98. **Marconi, R. T., M. E. Konkel, and C. F. Garon.** 1993. Variability of *osp* genes and gene products among species of Lyme disease spirochetes. Infect Immun **61:**2611-2617.

99. **Marconi, R. T., D. S. Samuels, R. K. Landry, and C. F. Garon.** 1994. Analysis of the distribution and molecular heterogeneity of the *ospD* gene among the Lyme disease spirochetes: evidence for lateral gene exchange. J Bacteriol **176:**4572-4582.

100. **Margolis, N., D. Hogan, K. Tilly, and P. A. Rosa.** 1994. Plasmid location of *Borrelia* purine biosynthesis gene homologs. J Bacteriol **176:**6427-6432.

101. **McDowell, J. V., J. Frederick, D. P. Miller, M. P. Goetting-Minesky, H. Goodman, J. C. Fenno, and R. T. Marconi.** 2011. Identification of the primary mechanism of complement evasion by the periodontal pathogen, *Treponema denticola*. Mol Oral Microbiol **26:**140-149.

102. **McDowell, J. V., M. E. Harlin, E. A. Rogers, and R. T. Marconi.** 2005. Putative coiled-coil structural elements of the BBA68 protein of Lyme disease spirochetes are required for formation of its factor H binding site. J Bacteriol **187:**1317-1323.

103. **McDowell, J. V., B. Huang, J. C. Fenno, and R. T. Marconi.** 2009. Analysis of a unique interaction between the complement regulatory protein factor H and the periodontal pathogen *Treponema denticola*. Infect Immun **77:**1417-1425.

104. **McDowell, J. V., J. Lankford, L. Stamm, T. Sadlon, D. L. Gordon, and R. T. Marconi.** 2005. Demonstration of factor H-like protein 1 binding to *Treponema denticola*, a pathogen associated with periodontal disease in humans. Infect Immun **73:**7126-7132.

105. **McDowell, J. V., S. Y. Sung, M. Labandeira-Rey, J. T. Skare, and R. T. Marconi.** 2001. Analysis of mechanisms associated with loss of infectivity of clonal populations of *Borrelia burgdorferi* B31MI. Infect Immun **69:**3670-3677.

106. **McDowell, J. V., E. Tran, D. Hamilton, J. Wolfgang, K. Miller, and R. T. Marconi.** 2003. Analysis of the ability of spirochete species associated with relapsing fever, avian borreliosis, and epizootic bovine abortion to bind factor H and cleave C3b. J Clin Microbiol **41:**3905-3910.

107. **McDowell, J. V., J. Wolfgang, L. Senty, C. M. Sundy, M. J. Noto, and R. T. Marconi.** 2004. Demonstration of the involvement of outer surface protein E coiled coil structural domains and higher order structural elements in the binding of infection-induced antibody and the complement-regulatory protein, factor H. J Immunol **173:**7471-7480.

108. **McDowell, J. V., J. Wolfgang, E. Tran, M. S. Metts, D. Hamilton, and R. T. Marconi.** 2003. Comprehensive analysis of the factor H binding capabilities of *Borrelia* species associated with lyme disease: delineation of two distinct classes of factor H binding proteins. Infect Immun **71:**3597-3602.

109. **Mehra, R., D. Londono, M. Sondey, C. Lawson, and D. Cadavid.** 2009. Structure-function investigation of Vsp serotypes of the spirochete *Borrelia hermsii*. PLoS One **4:**e7597.

110. **Meri, S., and H. Jarva.** 2008. Complement Regulatory Proteins, In: Encyclopedia of Life Sciences (ELS).DOI: 10.1002/9780470015902.a0001464.pub2.

111. **Meri, S., and M. K. Pangburn.** 1990. Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. P Natl Acad Sci USA **87:**3982-3986.

112. **Meri, T., A. Hartmann, D. Lenk, R. Eck, R. Wurzner, J. Hellwage, S. Meri, and P. F. Zipfel.** 2002. The yeast *Candida albicans* binds complement regulators factor H and FHL-1. Infect Immun **70:**5185-5192.

113. **Meri, T., T. S. Jokiranta, J. Hellwage, A. Bialonski, P. F. Zipfel, and S. Meri.** 2002. *Onchocerca volvulus* microfilariae avoid complement attach by direct binding of factor H. J Infect Dis **185:**1786-1793.

114. **Negussie, Y., D. G. Remick, L. E. DeForge, S. L. Kunkel, A. Eynon, and G. E. Griffin.** 1992. Detection of plasma tumor necrosis factor, interleukins 6, and 8 during the Jarisch-Herxheimer reaction of relapsing fever. J Exp Med **175:**1207-1212.

115. **Newman, K., Jr., and R. C. Johnson.** 1984. T-cell-independent elimination of *Borrelia turicatae*. Infect Immun **45:**572-576.

116. **Nordstrand, A., I. Bunikis, C. Larsson, K. Tsogbe, T. G. Schwan, M. Nilsson, and S. Bergstrom.** 2007. Tickborne relapsing fever diagnosis obscured by malaria, Togo. Emerg Infect Dis **13:**117-123.

117. **Nordstrand, A., A. Shamaei-Tousi, A. Ny, and S. Bergstrom.** 2001. Delayed invasion of the kidney and brain by *Borrelia crocidurae* in plasminogen-deficient mice. Infect Immun **69:**5832-5839.

118. **Nordstrom, T., A. M. Blom, A. Forsgren, and K. Riesbeck.** 2004. The emerging pathogen *Moraxella catarrhalis* interacts with complement inhibitor C4b binding protein through ubiquitous surface proteins A1 and A2. J Immunol **173:**4598-4606.

119. **Norris, S. J.** 2006. Antigenic variation with a twist--the *Borrelia* story. Mol Microbiol **60:**1319-1322.

120. **Pangburn, M. K., and H. J. Muller-Eberhard.** 1980. Relation of a putative thioester bond in C3 to activation of the alternative pathway and the binding of C3b to biological targets of complement. J Exp Med **152:**1102-1114.

121. **Pausa, M., V. Pellis, M. Cinco, P. G. Giulianini, G. Presani, S. Perticarari, R. Murgia, and F. Tedesco.** 2003. Serum-resistant strains of *Borrelia burgdorferi* evade complement-mediated killing by expressing a CD-59-like complement inhibitory molecule. J Immunol **170:**3214-3222.

122. **Porcella, S. F., S. J. Raffel, D. E. Anderson, Jr., S. D. Gilk, J. L. Bono, M. E. Schrumpf, and T. G. Schwan.** 2005. Variable tick protein in two genomic groups of the relapsing fever spirochete *Borrelia hermsii* in western North America. Infect Immun **73:**6647-6658.

123. **Potempa, M., J. Potempa, M. Okroj, K. Popadiak, S. Eick, K. A. Nguyen, K. Riesbeck, and A. M. Blom.** 2008. Binding of complement inhibitor C4b-binding protein contributes to serum resistance of *Porphyromonas gingivalis*. J Immunol **181:**5537-5544.

124. **Purser, J. E., and S. J. Norris.** 2000. Correlation between plasmid content and infectivity in *Borrelia burgdorferi*. P Natl Acad Sci USA **97:**13865-13870.

125. **Ram, S., A. K. Sharma, and S. D. Simpson.** 1998. A novel sialic acid binding site on factor H mediates serum resistance of non-sialylated *Neisseria gonorrhoeae*. J Exp Med **187:**743-752.

126. **Raoult, D., and V. Roux.** 1999. The body louse as a vector of reemerging human diseases. Clin Infect Dis **29:**888-911.

127. **Ras, N., B. Lascola, D. Postic, S. J. Cutler, F. Rodhain, G. Baranton, and D. Raoult.** 1996. Phylogenesis of relapsing fever *Borrelia* spp. Int J Syst Bacteriol **46:**859-865.

128. **Rautemaa, R., G. A. Jarvis, P. Marnila, and S. Meri.** 1998. Acquired resistance of *Escherichia coli* to complement lysis by binding of glycophosphoinositol-anchored protectin (CD59). Infect Immun **66:**1928-1933.

129. **Rawlings, J. A.** 1995. An overview of tick-borne relapsing fever with emphasis on outbreaks in Texas. Tex Med **91:**56-59.

130. **Rebaudet, S., and P. Parola.** 2006. Epidemiology of relapsing fever borreliosis in Europe. FEMS Immunol Med Microbiol **48:**11-5.

131. **Rogers, E. A., and R. T. Marconi.** 2007. Delineation of species-specific binding properties of the CspZ protein (BBH06) of Lyme disease spirochetes: evidence for new contributions to the pathogenesis of *Borrelia* spp. Infect Immun **75:**5272-5281.

132. **Rosa, P. A., K. Tilly, and P. E. Stewart.** 2005. The burgeoning molecular genetics of the Lyme disease spirochaete. Nat Rev Microbiol **3:**129-143.

133. **Ross, P. H., and A. D. Milne.** 1904. Tick fever. BMJ **2:**1453-1454.

134. **Rossmann, E., P. Kraiczy, P. Herzberger, C. Skerka, M. Kirschfink, M. M. Simon, P. F. Zipfel, and R. Wallich.** 2007. Dual binding specificity of a *Borrelia hermsii*-associated complement regulator-acquiring surface protein for factor H and plasminogen discloses a putative virulence factor of relapsing fever spirochetes. J Immunol **178:**7292-7301.

135. **Ryckman, R. E., and G. M. Kohls.** 1962. The desert tortoise, *Gopherus agassizi*, a host for the tick *Ornithodoros turicata* in California. J Parasitol **48:**502-503.

136. **Samuels, D. S., K. Mach, and C. F. Garon.** 1994. Genetic transformation of the Lyme disease agent *Borrelia burgdorferi* with coumarin-resistant *gyrB*. J Bacteriol **176:**6045-6049.

137. **Sarih, M., and e. al.** 2009. *Borrelia hispanica* relapsing fever, Morocco. Emerg Infect Dis **15:**1626-1629.

138. **Sarma, J. V., and P. A. Ward.** 2011. The complement system. Cell Tissue Res **343:**227-235.

139. **Schmidt, C. Q., A. P. Herbert, D. Kavanagh, C. Gandy, C. J. Fenton, B. B. Blaum, M. Lyon, D. Uhrin, and P. N. Barlow.** 2008. A new map of glycosaminoglycan and C3b binding sites on factor H. J Immunol **181:**2610-2619.

140. **Schneider, M. C., B. E. Prosser, J. J. Caesar, E. Kugelberg, S. Li, Q. Zhang, S. Quoraishi, J. E. Lovett, J. E. Deane, R. B. Sim, P. Roversi, S. Johnson, C. M. Tang, and S. M. Lea.** 2009. *Neisseria meningitidis* recruits factor H using protein mimicry of host carbohydrates. Nature **458:**890-893.

141. **Schott, M., S. Grosskinsky, C. Brenner, P. Kraiczy, and R. Wallich.** 2010. Molecular characterization of the interaction of *Borrelia parkeri* and *Borrelia turicatae* with human complement regulators. Infect Immun **78:**2199-2208.

142. **Schwan, T. G., W. Burgdorfer, and C. F. Garon.** 1988. Changes in infectivity and plasmid profile of the Lyme disease spirochete, *Borrelia burgdorferi*, as a result of in vitro cultivation. Infect Immun **56:**1831-1836.

143. **Schwan, T. G., and J. Piesman.** 2002. Vector interactions and molecular adaptations of lyme disease and relapsing fever spirochetes associated with transmission by ticks. Emerg Infect Dis **8:**115-121.

144. **Schwan, T. G., P. F. Policastro, Z. Miller, R. L. Thompson, T. Damrow, and J. E. Keirans.** 2003. Tick-borne relapsing fever caused by *Borrelia hermsii* in Montana. Emerg Infect Dis **9:**1151-1154.

145. **Schwan, T. G., S. J. Raffel, M. E. Schrumpf, J. S. Gill, and J. Piesman.** 2009. Characterization of a novel relapsing fever spirochete in the midgut, coxal fluid, and salivary glands of the bat tick *Carios kelleyi*. Vector-Borne Zoonot **9:**643-647.

146. **Schwan, T. G., S. J. Raffel, M. E. Schrumpf, P. F. Policastro, J. A. Rawlings, R. S. Lane, E. B. Breitschwerdt, and S. F. Porcella.** 2005. Phylogenetic analysis of the spirochetes *Borrelia parkeri* and *Borrelia turicatae* and the potential for tick-borne relapsing fever in Florida. J Clin Microbiol **43:**3851-3859.

147. **Schwan, T. G., S. J. Raffel, M. E. Schrumpf, and S. F. Porcella.** 2007. Diversity and distribution of *Borrelia hermsii*. Emerg Infect Dis **13:**436-442.

148. **Schwan, T. G., S. J. Raffel, M. E. Schrumpf, L. S. Webster, A. R. Marques, R. Spano, M. Rood, J. Burns, and R. Hu.** 2009. Tick-borne relapsing fever and *Borrelia hermsii*, Los Angeles County, California, USA. Emerg Infect Dis **15:**1026-1031.

149. **Scragg, I. G., D. Kwiatkowski, and V. Vidal.** 2000. Structural characterization of the inflammatory moiety of a variable major lipoprotein of *Borrelia recurrentis*. J Bio Chem **275:**937-941.

150. **Sethi, N., M. Sondey, Y. Bai, K. S. Kim, and D. Cadavid.** 2006. Interaction of a neurotropic strain of *Borrelia turicatae* with the cerebral microcirculation system. Infect Immun **74:**6408-6418.

151. **Shamaei-Tousi, A., O. Collin, A. Bergh, and S. Bergstrom.** 2001. Testicular damage by microcirculatory disruption and colonization of an immune-privileged site during *Borrelia crocidurae* infection. J Exp Med **193:**995-1004.

152. **Shamaei-Tousi, A., P. Martin, A. Bergh, N. Burman, T. Brannstrom, and S. Bergstrom.** 1999. Erythrocyte-aggregating relapsing fever spirochete *Borrelia crocidurae* induces formation of microemboli. J Infect Dis **180:**1929-1938.

153. **Singh, B., Y.-C. Su, and K. Riesbeck.** 2010. Vitronectin in bacterial pathogenesis: a host protein used in complement escape and cellular invasions. Mol Microbiol **78:**565-560.

154. **Southern, P. M., and J. P. Sanford.** 1969. Relapsing fever: A clinical and microbiological review. Medicine **48:**129-149.

155. **Spiller, G. W.** 1986. Tick-borne relapsing fever due to *Borrelia hermsii* in British Columbia. Can Med Assoc J **134:**46-47.

156. **Stanek, G., and F. Strle.** 2003. Lyme borreliosis. Lancet **362:**1639-1647.

157. **Steere, A. C., R. L. Grodzicki, A. N. Kornblatt, J. E. Craft, A. G. Barbour, W. Burgdorfer, G. P. Schmid, E. Johnson, and S. E. Malawista.** 1983. The spirochetal etiology of Lyme disease. N Engl J Med **308:**733-740.

158. **Steere, A. C., S. E. Malawista, D. R. Snydman, R. E. Shope, W. A. Andiman, M. R. Ross, and F. M. Steele.** 1977. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum **20:**7-17.

159. **Stevenson, B., H. A. Choy, M. Pinne, M. L. Rotondi, M. C. Miller, E. DeMoll, P. Kraiczy, A. E. Cooley, T. P. Creamer, M. A. Suchard, C. A. Brissette, A. Verma, and D. A. Haake.** 2007. *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin, and regulators of complement. PLoS One**:**e1188.

160. **Stewart, P. E., R. Byram, D. Grimm, K. Tilly, and P. A. Rosa.** 2005. The plasmids of *Borrelia burgdorferi*: essential genetic elements of a pathogen. Plasmid **53:**1-13.

161. **Stoenner, H. G., T. Dodd, and C. Larsen.** 1982. Antigenic variation of *Borrelia hermsii*. J Exp Med **156:**1297-1311.

162. **Takano, A., et al.** 2009. Relapsing fever spirochete in seabird tick, Japan. Emerg Infect Dis **15:**1528-1529.

163. **Takayama, K., R. J. Rothenberg, and A. G. Barbour.** 1987. Abscence of lipopolysaccharide in the Lyme disease spirochete, *Borrelia burgdorferi*. Infect Immun **55:**2311-2313.

164. **Talbert, A., A. Nyange, and F. Molteni.** 1998. Spraying tick-infested houses with lambda-cyhalothrin reduces the incidence of tick-borne relapsing fever in children under five years old. T Roy Soc Trop Med H **92:**251-253.

165. **Thern, A., L. Stenberg, B. Dahlback, and G. Lindahl.** 1995. Ig-binding surface proteins of *Streptococcus pyogenes* also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. J Immunol**:**375-386.

166. **Tilly, K., A. F. Elias, J. L. Bono, P. E. Stewart, and P. Rosa.** 2000. DNA exchange and insertional inactivation in spirochetes. J Mol Microbiol Biotechnol **2:**433-442.

167. **Trevejo, R. T., M. E. Schriefer, K. L. Gage, T. J. Safranek, K. A. Orloski, W. J. Pape, J. A. Montenieri, and G. L. Campbell.** 1998. An interstate outbreak of tick-borne relapsing fever among vacationers at a rocky mountain cabin. Am J Trop Med Hyg **58:**743-747.

168. **Vial, L., G. Diatta, A. Tall, H. Ba el, H. Bouganali, P. Durand, C. Sokhna, C. Rogier, F. Renaud, and J. F. Trape.** 2006. Incidence of tick-borne relapsing fever in west Africa: longitudinal study. Lancet **368:**37-43.

169. **Vial, L., P. Durand, C. Arnathau, L. Halos, G. Diatta, J. F. Trape, and F. Renaud.** 2006. Molecular divergences of the *Ornithodoros sonrai* soft tick species, a vector of human relapsing fever in West Africa. Microbes Infect **8:**2605-2611.

170. **Vidal, V., I. G. Scragg, S. J. Cutler, K. A. Rockett, D. Fekade, D. Warrell, D. J. M. Wright, and D. Kwiatkowski.** 1998. Variable major lipoprotein is a principle TNF-inducing factor of louse-borne relapsing fever. Nat Med **4:**1416-1420.

171. **Walker, R. L., D. H. Read, D. C. Hayes, and R. W. Nordhausen.** 2002. Equine abortion associated with the *Borrelia parkeri-Borrelia turicatae* tick-borne relapsing fever spirochete group. J Clin Microbiol **40:**1558-1562.

172. **Walport, M. J.** 2001. Complement- First of Two Parts. N Engl J Med **344:**1058-1066.

173. **Xu, Y., S. V. L. Narayana, and J. E. Volanakis.** 2001. Structural biology of the alternative pathway convertase. Immunol Rev **180:**123-135.

174. **Young, J. D.** 1998. Underreporting of Lyme disease. N Engl J Med **338:**1629.

175. **Zipfel, P. F., and C. Skerka.** 2009. Complement regulators and inhibitory proteins. Nat Rev Immunol **9:**729-740.

176. **Zipfel, P. F., C. Skerka, J. Hellwage, S. T. Jokiranta, S. Meri, V. Brade, P. Kraiczy, M. Noris, and G. Remuzzi.** 2002. Structure-function studies of the complement system. Biochem Soc T **30:**971-978.

Vita

Lindy Fine was born on September 10, 1979 in Washington, DC. After graduating from Bowie High School in 1997, she began studies as a Maryland Distinguished Scholar at St. Mary's College of Maryland. She received her Bachelor of Arts in Philosophy from St. Mary's in 2002.

Publications:

Lindy M. Fine, Christopher G. Earnhart, and Richard T. Marconi. Genetic transformation of the relapsing fever spirochete *Borrelia hermsii*: stable integration and expression of green fluorescent protein from linear plasmid 200 (lp200). J Bacteriol **193:** 3241-3245.